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α(1,3)galactosyl epitopes (αGal) expressed on membrane glycoproteins and glycolidpids are the major target of the human hyperacute rejection response observed when organs are transplanted from nonprimate donor species. Expression of the α(1,3)galactosyltransferase gene [α(1,3)GT] by breast cancer cells will lead to their direct in vivo destruction by anti-αGal antibodies and complement, and boost the immune response to other tumor antigens. In vitro transduction of MCF-7, and T47D human breast cancer cells with an HSV amplicon vector resulted in expression of the suicide gene as detected by specific binding of labeled IB-4 isolectin. Transduced breast cancer cells were susceptible to antibody and complement-mediated cyolysis as demonstrated in serum killing assays with 50% human serum. The α(1,3)GT knockout mouse is analogous to humans in terms of expression of αGal epitodpes. A syngeneic murine breast cancer cell line form α(1,3)GT knockout mice has been produced by chemical induction (BR340) that is being used to demonstrate protective immunity when cells are ex vivo transduced and implanted into αGal stimulated knockout mice.

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INTRODUCTION

From 1999 to 2001, an estimated 125,500 women have died from breast cancer despite all currently available therapies (1-3). New approaches are urgently needed to combat this deadly disease. Gene therapy is among the current experimental strategies for patients with cancer who have failed standard therapy, yet evidence for success in the more than 100 gene therapy trials to date is limited (4). New candidate genes and delivery methods must be pursued. This research exploits the α(1,3) galactosyltransferase [α(1,3)GT] gene for cancer therapy by converting transduced breast cancer cells into vaccines to stimulate systemic anti-cancer immunity. Strong immunologic reaction to xenotransplants from lower mammals into humans is based upon immunity to the α(1,3) GT gene product. Rejection of organ and tissue xenotransplants (5) occurs because pre-existing antibodies (Ab) in human serum to the α (1,3) Galactosyl epitope (α Gal) exist (6) and represent nearly 1% of total human serum Ab (7). Reacting antibodies activate complement resulting in hyperacute rejection of xenotransplants (8). The strategy of this research is to use the murine α(1,3)GT gene as a therapeutic transgene to induce hyperacute rejection of human breast cancer like that associated with xenotransplants. Our novel HSV amplicon vectors offer highly efficient gene transfer and expression of the α(1,3)GT gene. Herpes vectors efficiently transduce nondividing cells (G_0 stage) and the majority of human breast tumor cells are not actively dividing. In addition, these vectors transduce a wide variety of human adenocarcinomas. The research plan is listed below.

Statement of Work

Task 1. Demonstrate efficacy of HE7αgal1 amplicon vector in human breast cancer cells (months 1 to 18)

- 1a. Transduce human breast cancer cells with HE7αgal1 vector and demonstrate αgal epitope expression on the cell surface. (months 1 to 12)
- 1b. Demonstrate human breast cancer cell sensitivity to human serum after HE7αgal1 vector transduction and that the cytotoxocity is secondary to complement activation. (months 7 to 12)
- 1c. Complete and publish manuscript concerning *in vitro* transduction and complement mediated destruction of human breast cancer cells. (months 12 to 18)

Task 2. Demonstrate efficacy *in vivo* of HE7αgal1 vector in a murine breast cancer model in α(1,3)GT knockout mice. (months 1 to 36)

- 2a. Generate syngeneic breast tumor cell lines derived from α(1,3)GT knockout mcie. (months 1 to 9)
- 2b. Generate protective immunity against breast tumor cell challenge by preimmunization with *ex vivo* HE7αgal1 vector transduced breast cancer cells. (months 10 to 18)
- 2c. Destroy subcutaneous breast cancer tumors by direct injection of HE7αgal1 vector *in vivo*. (months 18 to 30)
- 2d. Evaluate humoral and cellular immune responses to αgal epitope presentation by breast tumors. (months 20 to 36)
- 2e. Complete and publish manuscript concerning *in vivo* immunization and protection using HE7αgal1 transduced murine breast cells derived from α(1,3)GT knockout mice. (months 24 to 36)

BODY

Task 1. Demonstrate efficacy of HE7αgal1 amplicon vector in human breast cancer cells.

1a. Transduce human breast cancer cells with HE7αgal1 vector and demonstrate αgal epitope expression on the cell surface.

Status: Completed

MCF-7 human breast adenocarcinoma cells, T47D human breast ductal carcinoma cells, and SKBR3 human breast adenocarcinoma cells were selected for testing the *in vitro* efficacy of the HE7αgal1 amplicon vector. All cells were maintained at 37°C in a 5% CO_2 incubator. The growth medium for MCF-7 cells consisted of Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen-Life Technologies, Carlsbad, CA) supplemented with bovine insulin (0.1 mg/ml) (Sigma, St. Louis, MO), and 10% FBS (Invitrogen-Life Technologies). T47D cells were maintained in RPMI 1640 medium (Invitrogen-Life Technologies) supplemented with bovine insulin (0.1 mg/ml) (Sigma), 0.1 mM nonessential amino acids (Sigma), 0.45% Glucose, and 10% FBS (Invitrogen-Life Technologies). SKBR3 cells were maintained in McCoys 5A medium (Invitrogen-Life Technologies) supplemented with 10% FBS (Invitrogen-Life Technologies), and 10% conditioned medium.

CgalΔ3 helper herpes virus is deleted for the IE3 gene and was propagated on the permissive cell line E5 (African Green Monkey Kidney), which contains a stably integrated IE3 gene. For propagation, 90% confluent cultures of E5 cells were infected with 0.1 moi of CgalΔ3 helper virus. Following incubation at 37°C for 72 hr, cells were harvested and subjected to three rounds of freeze/thawing. Virus was concentrated by centrifugation at 28,000 rpm for 1 hr and resuspended in 10% sucrose. Packaged HE7αgal1 amplicon was produced by transfecting the amplicon into E5 cells prior to CgalΔ3 propagation. Both helper virus and HE7αgal1 amplicon were titered on VA13 human fibroblast cells.

Breast cancer cells were cultured overnight in a 24 well plate (Corning Co., Corning, NY). Cells were washed and infected with HE7αgal1 vector at 3 moi in 200 µl of Opti-MEM (Invitrogen-Life Technologies). Plates were rocked to mix for 2 hr, and 300 µl of complete medium was added. Lectin staining for αGal epitopes, or serum killing assays were performed 24 hr post-transduction.

HE7αgal1 transduced tumor cells were stained with *Griffonia simplicifolia* (Vector Laboratories, Burlingame, CA) IB_4 isolectin conjugated to fluorescein isothiocyanate (FITC). α(1,3)Gal epitopes are

specifically bound by the GS IB_4 isolectin. Transduced cells were washed with 1x HBSS (Invitrogen-Life Technologies), then incubated under dark conditions at room temperature with 200 µl of a 10µl/ml solution of GS IB_4 isolectin diluted in 1x OptiMEM (Invitrogen-Life Technologies) for 10 minutes. Cells were washed with 1x HBSS, and viewed under 488 nm wavelength light. Labeled cells were observed for lectin binding using a Nikon Diaphot 300 Fluorescent microscope (Nikon Inc. Melville, NY), and photographed using a Nikon CoolPix995 digital camera (Nikon Inc.).

MCF-7, T47D, and SKBR3 human breast cancer cells were all successfully transduced by HE7αgal1 amplicon vector, and expression of αGal epitopes on surface proteins was detected by FITC- IB_4 isolectin staining (Fig 1).

1b. Demonstrate human breast cancer cell sensitivity to human serum after HE7αgal1 vector transduction and that the cytotoxicity is secondary to complement activation.

Status: Completed

Propagated HE7αgal1 vector was treated with psoralen and UVA light (PUVA) to reduce helper virus cytotoxicity but retain high transgene expression prior to use in assays. Psoralens are polycyclic planar molecules that form covalent, cyclobutane-type linkages. Previous studies applying crosslinking methods with psoralen and UVA completely inactivated virus. The appropriate PUVA dose induces DNA crosslinks in the vector and inhibits replication in E5 cells while retaining reporter gene expression. Psoralen was diluted to 1 µg/ml in HBSS and mixed with HE7αgal1 vector at room temperature for 30 minutes. Vector was then exposed to UVA ($2Kj/m^2$) for 5.5 minutes. Vector was then titered on VA13 cells as before.

Serum killing assays were performed in 96-well round bottom tissue culture plates. Transduced cancer cell lines were trypsinized, concentrated by centrifugation, and suspended in Opti-MEM. Cells were counted using Trypan Blue Exclusion, and 50 µl containing 1×10^6 cells was seeded into wells. Frozen human serum was thawed on ice and added 1:1 with cell suspensions to create a 50% dilution of human serum with transduced cancer cells, and cells were incubated for 1 hr at 37°C in a 5% CO_2 incubator. Cells were transferred to a flow cytometry culture tube, washed with 4.0 ml of Opti-MEM, and incubated with GS IB_4 isolectin (FITC) and Propidium iodide for 15 minutes under dark conditions. Cells were washed with 4.0 ml of HBSS, and suspended in 1.0 ml of HBSS for analysis by flow cytometry. Flow

cytometry was performed using a Coulter Epics Altra Flow Cytometer (Coulter Corp, Miami, FL) for two color analysis, gated for approximately 10,000 events. Human serum killing of transduced human breast cancer cell lines is shown in Fig 3.

1c. Complete and publish manuscript concerning *in vitro* transduction and complement mediated destruction of human breast cancer cells.

Status: Manuscript in preparation.

Concurrent work using Adenoviral delivery of the gene for αgal to breast cancer cells, has been completed. The Adenovirus system offers many advantages over Herpesviral delivery of recombinant genes, including greater expression of gene products, and ease of recombinant virus production. *In vitro* serum killing assays identical to those described in Task 1b have been performed and repeated using a recombinant Ad-αGT virus created by this Institute. Breast tumor cell lines were infected, and 48h post-infection, cells were incubated with 50% normal human serum (NHS). Results of serum killing assays using MCF-7, BT-20, and T47D breast cancer cell lines are shown in figure 8. Further results using this recombinant Ad-αGT are discussed in meeting abstracts (appendix).

Task 2. Demonstrate efficacy *in vivo* of HE7αgal1 vector in murine breast cancer model in α(1,3)GT knockout mice.

2a. Generate sygeneic breast tumor cell lines derived from α(1,3)GT knockout mice.

Status: Completed

The αGal epitope is expressed in all mammals except humans and Old World primates. Lowe et al., produced an α (1,3)GT knockout strain of C57/BL6 mice by interrupting the α (1,3)GT gene (9). This mouse strain lacks a functional α (1,3) GT gene. It has been shown that these mice, like humans, produce anti-αgal antibody, albeit much less than in humans (10). When primed with rabbit red blood cells, which express approximately 10^6 αgal epitopes per cell, anti-αgal antibody titer rises to that of levels found in humans (11). The only murine tumor line known to be devoid of αgal expression was the C57/BL6 derived melanoma B16F0 and its derivatives (12). We induced the formation of tumors in the αgal knockout mice through multiple injections of 3-methylcholanthrene (3-MC), 9,10-dimethyl-1,2-benzanthracene (DMBA), and 6α-methyl-17β-hydroxy-progesterone acetate (MD) all dissolved in olive oil. Forty-four tumors were collected and approximately 50% were successfully cultured. Primary

histopathology demonstrated tumors ranging from squamous cell carcinoma of the dermis, to adenocarcinoma of the small intestine, and adenocarcinoma of the breast.

To confirm the absence of αgal expression, tumor cells were stained with the αgal epitope specific FITC-labeled IB_4 lectin. All cell lines tested were negative upon staining. Expression of αgal epitopes on these tumor cells is critically important if they are to be used as tumor vaccines. Furthermore, gene transfer by an HSV-1 viral vector had to be established in these cells in order to use this gene delivery vehicle in both *in vitro* and *in vivo* experiments. Therefore, The BR340 α(1,3)GT Knockout breast cancer cell line was transduced with HE7αgal1, and αgal epitope expression was measured by IB_4 lectin binding. This murine cell line expressed high levels of αgal epitopes (Fig 2).

Based upon findings described in Task 2b, we are in the process of developing new breast tumor cell lines derived from 4-6 wk old mice, by direct injection into the mammary fat pad with DMBA dissolved in olive oil. The protocol is identical with the exception of using only DMBA for injection of mice. The mice used for these experiments are of the b haplotype and not yet truly inbred, but we hope that new tumor cells that will be isolated and characterized will grow better in mice when re-implanted. Resulting tumors will be analyzed by histopathology, cultured in vitro, and serially passaged in vivo immediately following tumor harvest.

A second αgal-negative tumor cell line was generated during the initial experiments that yielded the BR340 cell line. This tumor cell line CA320M was characterized as a sarcoma of the small intestine (Reprint 1). This cell line was established in cell culture and analyzed (Figs 9, 10). These cells were shown to be homozygous for the $H\text{-}2^{b/b}$ haplotype and demonstrated no detectable cell surface αgal expression (Fig 11). *In Vitro* serum killing analyses of transduced CA320M cells demonstrates that αgal expressing cells are killed by exposure to NHS (Fig 12). And *in vivo* tumor growth characteristics generated as described in Task 2b, demonstrate that tumors grow when cells are implanted subcutaneously on αGT Knockout mice (Fig 13).

2b. Generate protective immunity against breast tumor cell challenge by preimmunization with *ex vivo* HE7αgal1 vector transduced breast cancer cells

Status: Continuing

In order to analyze protective immunity associated with agal in knock-out mice, we had to establish growth characteristics of the newly developed breast tumor line BR340. Determination of cell dose, volume and duration was vital to defining an in vivo model. However, we have spent much time in characterizing BR340with limited success.

After establishing BR340 in culture by multiple passage (greater than 30) we began to analyze the in vivo growth characteristics. Tumor cells were introduced subcutaneously at a concentration of $1x10^6$ per 100 µl, and $5x10^6$ per 100 µl in Hanks Balanced Salt Solution. It was noted that tumors developed after three days and continued to maximal size by day 10. Where upon the tumors began regressing. By day 25 all animals were devoid of tumors and remained that way for 90 days. During that time it was evident that the tumor cells appeared to be pleomorphic in culture, a common occurrence with DMBA induced tumors (13). In order to clone a subpopulation, we introduced $1x10^7$ cells per animal and conducted serial in vivo passages of resulting tumors. Each tumor was harvest before reaching 10 days, cultured and re-introduced into another animal. After five passages the tumor cells were expanded in culture and introduced into 8 mice. Unfortunately the response was similar, regression after 10 days in vivo.

We began a parallel investigation into the genetic background of the mice from which this tumor was derived, the same colony into which we were implanting BR340 cells. It became apparent that the transgenic knockout mice were not backcrossed to yield an inbred strain. Therefore, we found that the genetic background of C57/Bl6 x DBA/2 x 129SV produced to haplotypes of d or b. In lieu of that finding we haplotyped our tumor lines and nearly 400 mice from our colony. Anti-H2-K^d, Anti-H2-K^b, Anti-H2-D^b, Anti-H2-D^d antibodies (Pharmingen, San Diego, CA) were used on blood samples collect from the saphenous vein of mice. Also, these antibodies were used to analyze tumor cells by flow cytometry (Fig 4). We found that the BR340 tumor line belongs to the d haplotype. Furthermore, since the tumor was derived from a female mouse, it became apparent that tumor growth may be optimized in haplotype and sex match mice.

After several months of amplifying the colony, d haplotype female mice were used to assess the growth characteristics of BR340. Cells were introduced by subcutaneous injection as described above, and

followed for growth. Figure 5 demonstrates that the growth characteristics followed the same pattern as previously described.

In order to understand the basis behind the tumor rejection, a retroviral vector carrying the cytomegalovirus US11 gene was introduced to BR340 cells. US11 inhibits expression of the major histocompatibility complex I, a major mediator of graft rejection. Median fluorescence of BR340LUSN decreased by 74% from parental and 90% from the control (null retroviral vector, LXSN) (Fig 6). However, when the cells were introduced into haplotype and sex matched animals, the cells failed to grow past 10 days.

At this point we began to investigate several simultaneous approaches in order to develop this tumor model. First we subjected the cells to stringent serum starvation (SS) conditions. BR340 cells were trypsinized and re-seeded in a T-175 flask at a concentration of $2x10^6$ cells and allowed to attach overnight in complete media supplemented with 10% fetal bovine serum (FBS). The follow day the growth media was changed to complete media with 0.1% FBS and allowed to grow for 13 days. The media was changed to complete media with 2.0% FBS and allowed to grow for another 3 days whereupon the cells were further recovered in complete media with 10% FBS. In a parallel approach, BR340 was subject to 1mCu I^{131} for 24hrs which resulted in the killing of approximately 90% of the cells. The cells were recovered and subjected to 1.5mCu I^{131} for 48hrs. Approximately 98% of the cells were killed and the remaining cells were recovered in complete media.

Both of these modified cell lines were introduced into mice and followed for 90 days. All tumors but one regressed by day 14. Animal D708 received a subcutaneous injection of SS modified BR340 at $1x10^6$ cells. By day 30 the tumor was approximately $550mm^3$. We harvested the tumor and cut it in half for in vivo propagation and in vitro amplification. Five tumor fragments were introduced subdermally in matched mice and the remaining tumor was digested and culture in complete media. After a week amplification, the BR340.D708 cells were introduced by subcutaneous injection into the mammary fat pad of 8 matched mice and tumor growth was followed.

The implanted tumor fragments failed to grow larger tumors whereas the subcutaneous injections produced tumors that again regressed by day 14. A common observation with BR340 in vivo, as was true from the primary tumor, was that as the tumor grew the center became to necrotic. By day 10 noticeable cell death was observed as the tumor began to take on a gelatinous appearance. On the belief that the tumor may be dying because of a lack of growth factors, we investigated the use of a gel used to help establish growth of tumors in vitro and in vivo. We mixed MatriGel (BD BioSciences, Bedford, MA) with

BR340.D708 cells and injected as before. This time we noted sustained tumor growth to day 25 before regression began.

Further analyses by flow cytometry demonstrated that BR340 expresses the α chain of the estrogen receptor. We reasoned that since the tumor grew very well in tissue culture without a requirement for hormonal supplement, that growth in vivo may not be affected by supplemented estrogen. To prove that, we implanted estrogen tablets (1.6mg/tablet 60 day slow release) subdermally and 24hrs later injected BR340 cells as above. No gain in growth rate or duration was observed with this treatment.

We have attempted to clone individual subclones of BR340 by inducing spheroid formation on soft agar. Miller had demonstrated that drug induced tumors are often not monoclonal and that subclones can demonstrate immunodomminance over other subclones (13). Therefore, we thought it would be of some value to attempt to subclone this tumor line and investigate the subclones for in vivo growth. These subclones that were generated have not yet been tested for growth *in vivo*. Additionally, we further mutagenized BR340 cells using short wavelength UVC radiation. One million cells each were subject to various doses of UVC and plated in complete media. At 200J/m2, for example, only 4 colonies survived the treatment. Each treatment was repeated on the respective cells, and each population was introduced into 8 animals each. Fewer BR340 cells were needed to induce tumor formation. Previously, 2×10^7 cells injected with matrigel, were needed to induce a tumor that would regress by day 21. Using the UVC irradiated BR340 cells, a total of 5×10^6 cells without matrigel, were able to induce tumors of the same size. However, these tumors also regressed after 30 days (data not shown). Further experiments have not been attempted while we are waiting for new breast tumor cell lines to develop in recently bred mice injected with DMBA.

While we have been awaiting new breast tumor cell lines, we have initiated identical experiments using the CA320M cell line. Preliminary results indicate that natural tumor regression also occurs with this cell line. Conditions for tumor growth are being optimized at this time.

2c. Destroy subcutaneous breast cancer tumors by direct injection on HE7αgal1 vector *in vivo*

Status: On hold

Due to difficulties in growing our αgal-negative murine breast tumor cells *in vivo*, we have been forced to postpone these experiments until the new murine breast tumor cell lines are produced. Experiments using the currently available BR340 cell line would have been difficult to evaluate given the natural regression of tumors

over time. Newly derived cell lines from b haplotype mice that have been inbred for more generations will undoubtedly prove to be better models for destruction following direct injection of the HE7αgal1 vector.

We have demonstrated that the recombinant Ad-αGT virus can infect murine tumor cell lines (BR340, CA320M, and B16 melanoma) at relatively low MOI. *In vivo* animal experiments are planned to demonstrate destruction of subcutaneous murine tumors following direct injection of this virus into immunologically primed mice.

2d. Evaluate humoral and cellular immune responses to αgal epitope presentation by breast tumors

Status: Continuing

The humoral and cellular immune responses to αgal epitopes in breast tumors have not yet been completed due to difficulties in generation of αgal-negative tumor cell lines derived from αGT knockout mice as described in Task 2a. New αgal-negative breast tumors are being generated by chemical induction, and will be used to complete the work. Similar analysis of the immune response to αgal epitopes using a different model system, the B16 melanoma cell line, have been carried out concurrently. Although these are not results using breast tumor cells, the findings begin to shed light on the induction of immunity to αgal epitopes.

While we are awaiting new αgal-negative breast tumor cell lines, studies have been initiated using the B16 melanoma model. While this is not the model approved for this study, generated data and protocols will be useful for the future when the desired breast cell lines are available. And data are offered here to demonstrate proof of principle.

Immunologic Studies after vaccination with αGal expressing vaccines in the B16 melanoma model: Preliminary data obtained in our laboratory by Dr Rossi using B16 melanoma cells as a model in the αGT KO mouse is presented instead. Mice vaccinated with αGal expressing B16 cells were protected against lethal challenge with the αGal negative B16 melanoma cell line (not shown). In addition mice bearing pre-established subcutaneous and pulmonary melanoma tumors were treated efficiently by αGal expressing B16 cell vaccines (not shown). T cells harvested from mice protected after vaccination with αGal expressing B16 vaccines recognized *in vitro* B16 lacking the expression of αGal epitopes. These T cell precursors were absent in control mice (Fig 14). In a similar experiment it was demonstrated that these T cells precursors were specific for

melanoma since they were not able to recognize the αGal negative small intestine cell line CA320M (Fig 15). These results indicate that vaccination with αGal expressing whole cancer cells induce T cell immunity able to prevent and treat pre-established αGal negative tumors.

2e. Complete and publish manuscript concerning *in vivo* immunization and protection using HE7αgal transduced murine breast cancer cells derived from α(1,3)GT knockout mice.

Status: On hold

The manuscript showing protection using HE7αgal transduced murine breast cancer cells will be on hold until new cell lines are generated in our re-established (further cross-bred) colony.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that HE7αgal1 vector can effectively transduce human breast cancer cell lines (MCF-7; T47D; and SKBR3), and the generated murine breast cancer cell line (BR340).

- Demonstrated expression of the αGT transgene in breast cancer cell lines transduced with HE7αgal1 vector by specific binding of FITC-labeled IB_4 lectin and fluorescent microscopy.

- Demonstrated sensitivity of human and BR340 murine breast cancer cell lines to human serum after HE7αgal1 transduction using 50% human serum in an *in vitro* assay.

- Generated a syngeneic murine breast cancer cell line derived from α(1,3)GT knockout mice (BR340) using chemical induction with 3-MC, DMB, and MD.

- Generated a syngeneic murine gastrointestinal stromal tumor cell line derived from α(1,3)GT knockout mice (CA320M) using chemical induction.

- Generated other syngeneic murine cancer cell lines (small intestine, squamous cell carcinoma, and fibrosarcoma) that will be very useful in future work using αGT as a therapeutic gene for cancer therapy.

- Demonstrated the presence of complement-inhibitory factors that will need to be overcome in order to optimize αgal-mediated killing of tumor cells.

Key research accomplishments related to this study that will assist in fulfilling all state Tasks

- Generation of a recombinant Adenovirus carrying the αGT gene that infects human and murine tumor cell lines, and demonstration of αGT expression and killing by NHS.

- Generated immunologic data in the B16 melanoma model. Protocols developed for this model will be used for new breast tumor cell lines.

Determined potential new avenues of research based upon possible complement-inhibition and interference of serum killing of αGT expressing cancer cells.

REPORTABLE OUTCOMES

The research supported by this funding has generated the following reportable outcomes:

- A manuscript accepted by the journal Cancer Research "Immunity to the α(1,3)Galactosyl Epitope Provides Protection in Mice Challenged with Colon Cancer Cells Expressing α(1,3)Galactosyltransferase: A Novel Suicide Gene for Cancer Gene Therapy" (Reprint 2)

- A manuscript draft concerning generation and characterization of a sygeneic murine tumor cell line that can be used for development of cancer gene therapy protocols with the α(1,3)GT gene.

- A manuscript draft concerning a high-throughput fluorescent screening of transgenic mice. (Reprint 3)

- Abstracts and presentations at:

  Department of the Army – Era of Hope Meeting 2002
  Poster Presentation – A Novel Therapy for Breast Cancer by Inducing Hyperacute Rejection

  Komen Foundation "Reaching for the Cure – Innovations in Quality Care" 2001
  Oral Presentation – Breast Cancer Vaccine by Inducing Hyperacute Rejection

  2000 ASGT Annual Meeting – Eliciting Hyperacute Rejection of HSV α(1,3) Galactosyltransferase Transduced Tumors

  2000 ASGT Annual Meeting – Evidence for a Protective Immune Stimulation from α(1,3)Galactosyl Epitopes in Mice

  2001 ASGT Annual Meeting – Use of α(1,3)galactosyltransferase (αGT) as a Novel Suicide Gene for Cancer Gene Therapy

  2001 AACR Annual Meeting – Generation of a Recombinant Adenovirus Carrying the Gene for α(1,3)Galactosyl Transferase, For use as a Suicide Gene in Cancer Immunotherapy

  2002 AACR Annual Meeting – Suicide Gene Expression by an HSV Amplicon Vector Using a Complementation System of an IE3-deficient Herpesvirus with a Recombinant Adenovirus Expressing the HSV-ICP4 Gene in Co-infected Cells.

  2003 ASGT Annual Meeting – Characterization of a Gastrointestinal Stromal Tumor Line Derived from an α(1,3)galactosyltransferase Knockout Mouse and its Evaluation as a Potential Gene Therapy Model.

  2003 ASCO Annual Meeting – Adenoviral Gene Delivery of α(1,3)Galactosyltransferase to Breast Cancer Cells: Tumor Cell Killing by Innate Immunity.

  2003 ASGT Annual Meeting – Cancer Gene Therapy: Adenoviral Delivery of the Suicide Gene αGT Results in Tumor Cell Killing by Innate Immunity.

- Approval of two Phase I/II clinical trials based upon α(1,3)GT cancer gene therapy.

  0208-550 A Phase I/II Study of an Antitumor Vaccination using α(1,3)galactosyltransferase Expressing Allogeneic Tumor Cells in Patients with Relapsed or Refractory Breast Cancer

  0210-552 A Phase I/II Study of an Antitumor Vaccination using α(1,3)galactosyltransferase Expressing Allogeneic Tumor Cells in Patients with Refractory or Recurrent Non-Small Cell Lung Cancer

- Support of Dissertation Degree work for co-invetigator Daniel Hellrung. Graduation date, 12/2003

CONCLUSIONS

The work supported by this funding has demonstrated the possibility of using the α(1,3)GT transgene as a potent effector for cancer gene therapy. The HE7αgal HSV vector that we have produced is able to successfully transduce human breast cancer cell lines that we have tested, and can also transduce the generated syngeneic murine breast cancer cell line. This is an important factor necessary for destruction of transduced cells by human serum. Our results conclusively show that human serum will kill α(1,3)GT expressing tumor cells *in vitro*. We have also shown *in vivo* that an immune response to the αGal epitope in α(1,3)GT knockout mice can provide protection when mice are challenged with an αGal-positive tumor cell line (submitted manuscript). All findings to date have supported the hypothesis that the α(1,3)GT gene is an excellent candidate for gene therapy for breast cancer.

A major objective of this work, was the generation of a syngeneic breast tumor cell line that could be used in this work to further test our hypothesis. This goal was achieved, in addition to the generation of tumor cell lines from other murine tissues. These cell lines will be invaluable for future work in the characterization and refinement of α(1,3)GT cancer gene therapy.

Difficulties have been encountered while attempting to use the generated BR340 murine breast cancer cell line for generating *in vivo* tumors. These difficulties may be due to the low tumorigenicity of the cell line, or perhaps these cells are highly immunogenic. As stated, we are attempting to overcome this problem by further mutagenizing the cell line. In order to generate data that can be used in support of the fundamental hyposthesis, until difficulties in growing the BR340 cell line are solved, we are considering using one of the other generated syngeneic tumor cell lines. Figures 7 and 13 show the growth curve generated when the CA320M small intestine sarcoma cell line is injected subcutaneously into the α(1,3)GT knockout mouse. This tumor cell line is able to grow and generate subcutaneous tumors more readily, and is being used as an alternative model to produce data until the desired new reast tumor cell lines are available.

We have data and new research materials (cell lines) that supports the original hypothesis of using the α(1,3)GT gene for gene therapy for breast cancer. Data demonstrate that this hypothesis has a high potential for leading to an effective breast cancer gene therapy. The support of the Department of Defense has been instrumental in the generation of significant information leading to this goal.

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Figure 1.

Human Breast Carcinoma Transduced With HE7αgal1 and labeled with FITC IB_4 Isolectin

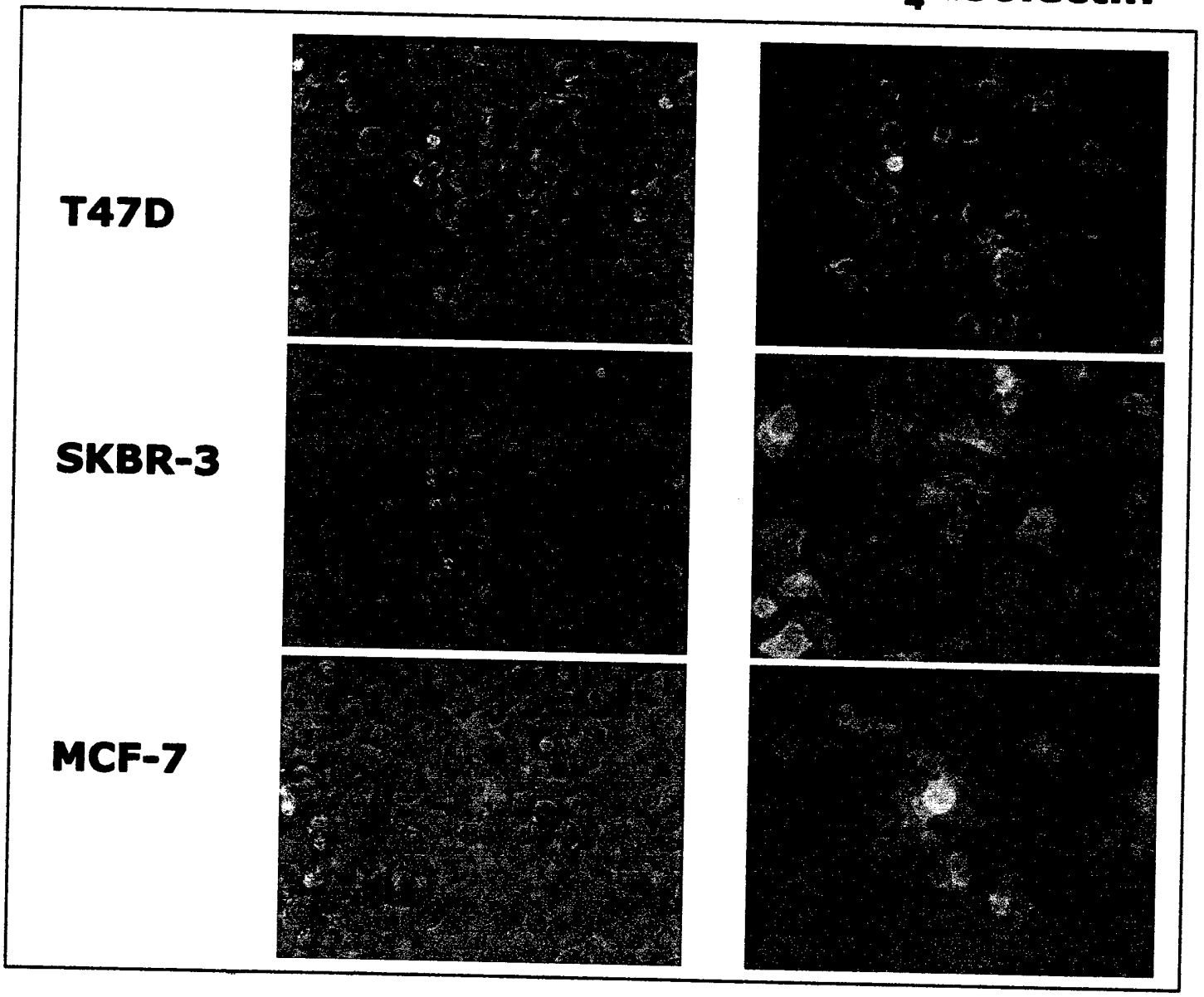


Figure 2.

Murine Breast Carcinoma Transduced With HE7αgal1 and labeled with FITC IB_4 Isolectin

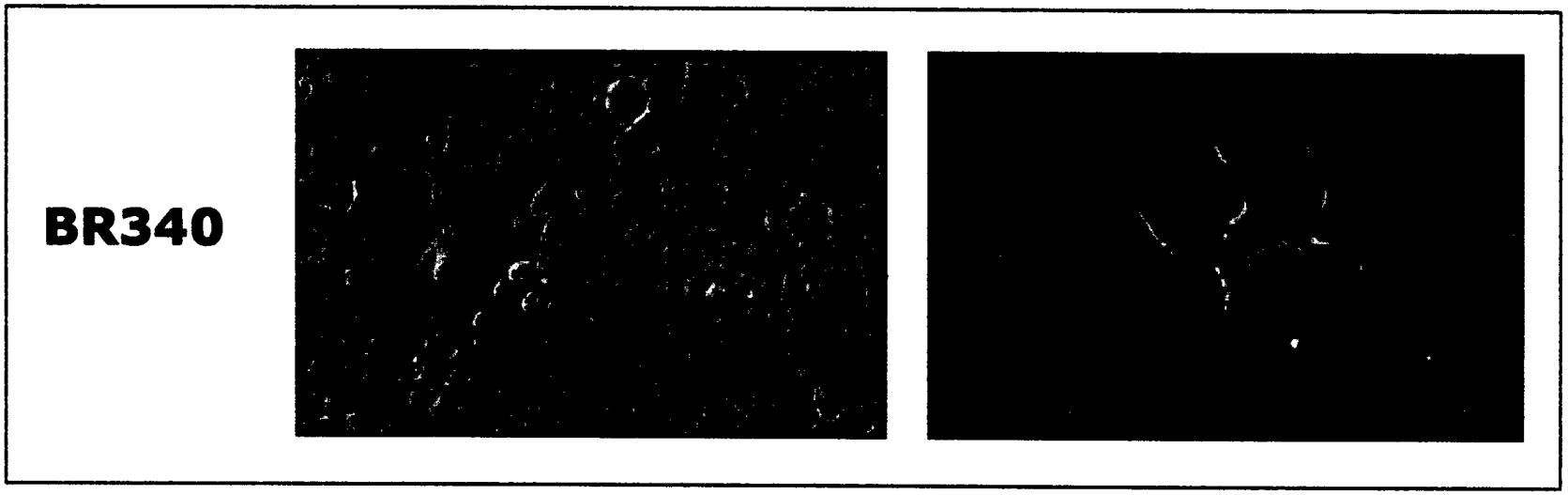


Figure 3

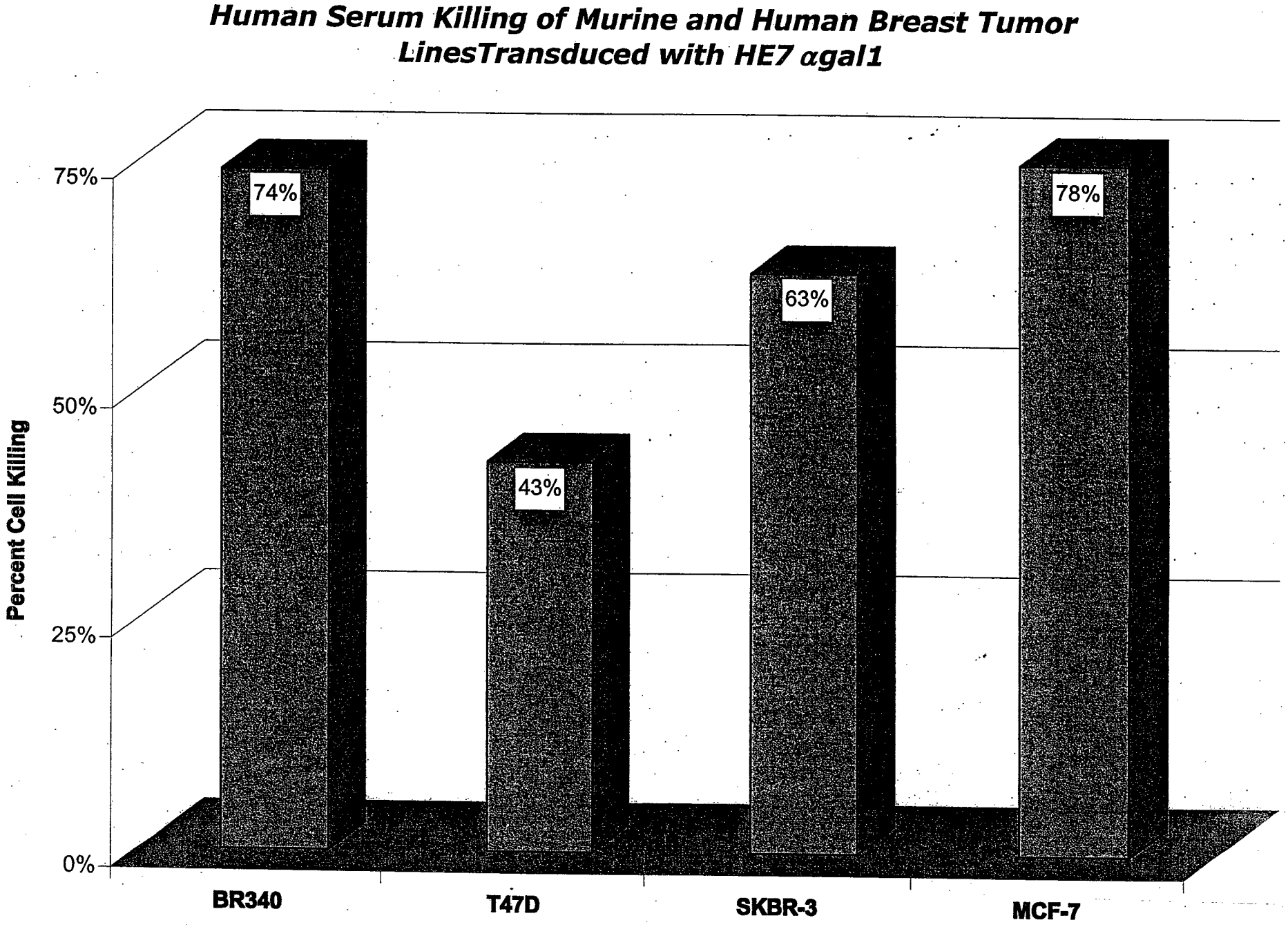

Human Serum Killing of Murine and Human Breast Tumor LinesTransduced with HE7 αgal1
Percent Cell Killing
75%
50%
25%
0%
74%
43%
63%
78%
BR340
T47D
SKBR-3
MCF-7

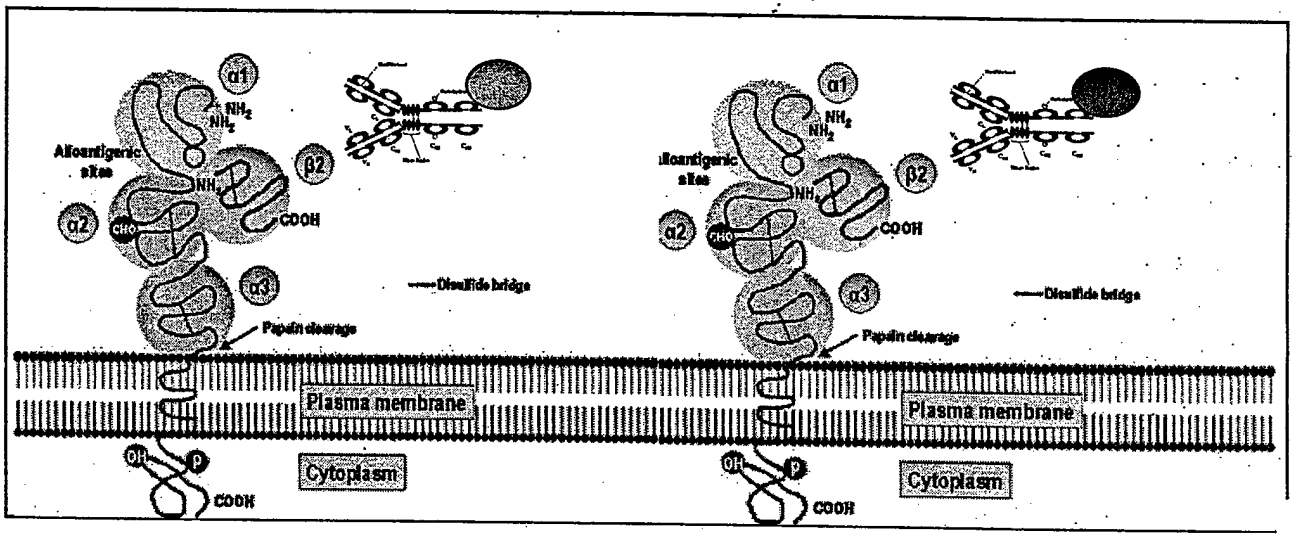
Figure 4.

Haplotyping

1. Isolate Blood

2. Label with FITC or PE conjugated anti-H2 Ab

3. HBSS Wash and View



Tumor	H2-K^b	H2-D^b	H2-K^d	H2-D^d
172	2	2	0	0
320M	2	2	0	0
260B	0	0	4	4
340	0	0	3	3

Figure 5

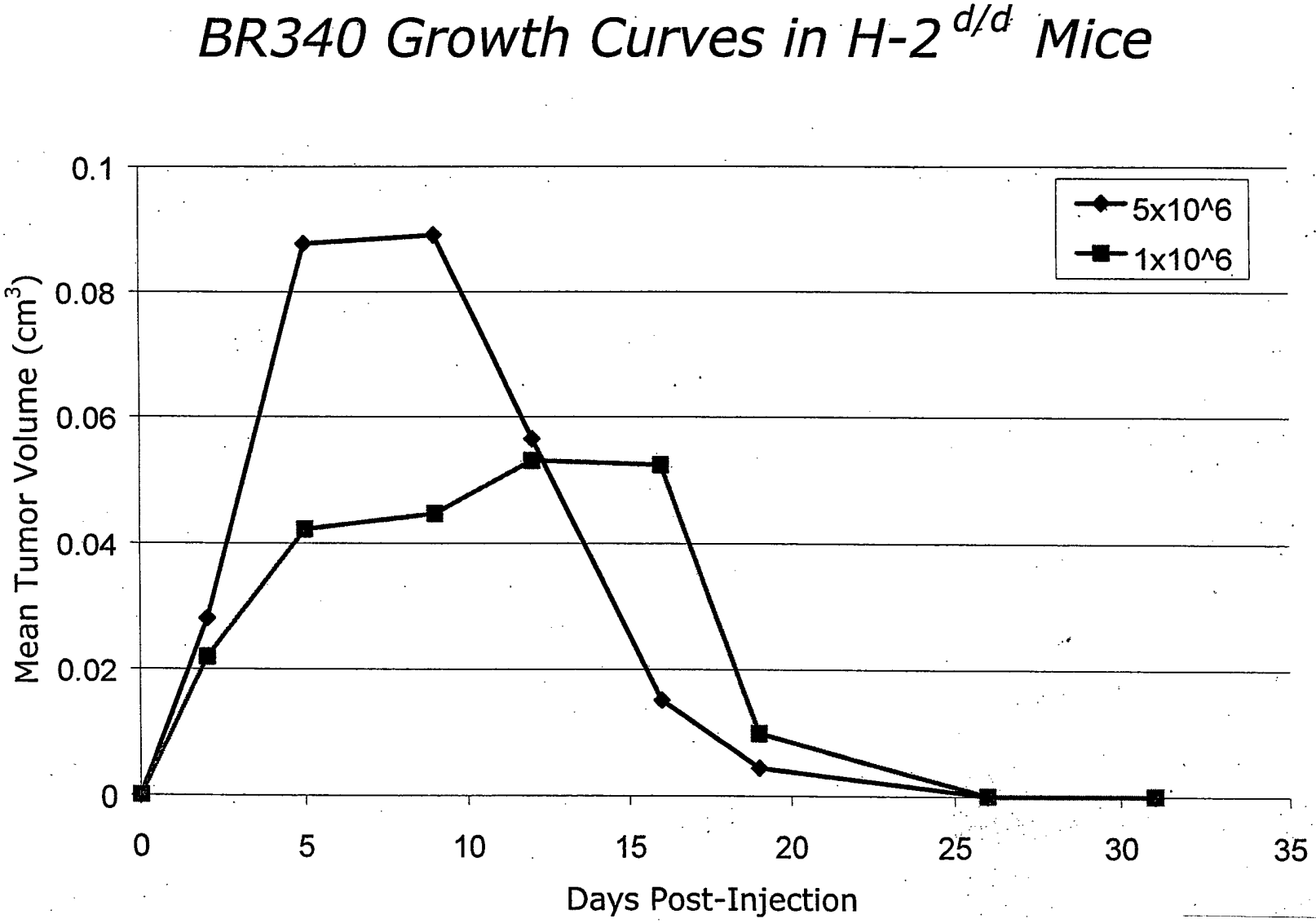

BR340 Growth Curves in H-2 d/d Mice
0.1
0.08
0.06
0.04
0.02
0
Mean Tumor Volume (cm3)
0
5
10
15
20
25
30
35
Days Post-Injection
5x10^6
1x10^6

Figure 6.

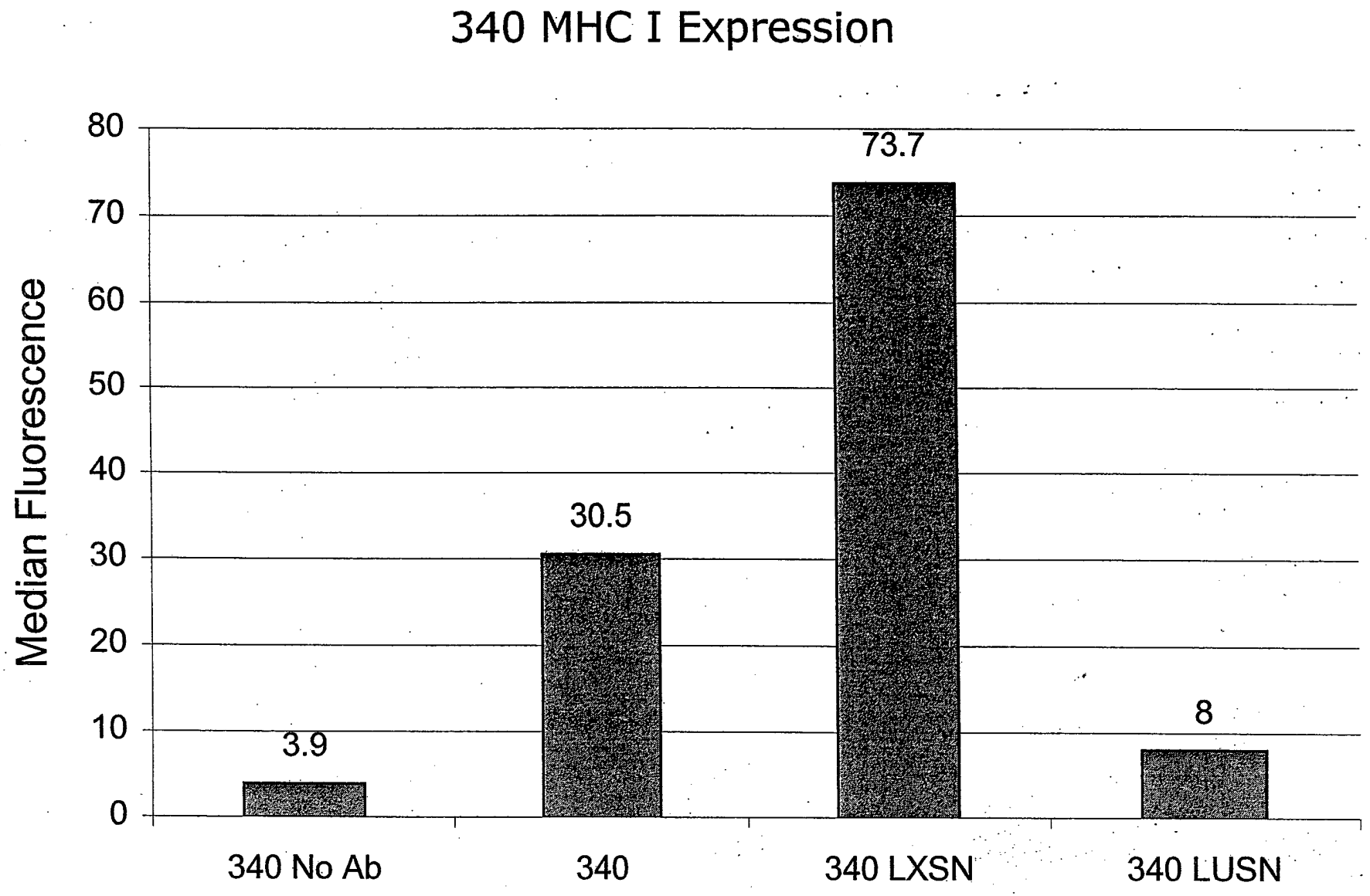

340 MHC I Expression
Median Fluorescence
80
70
60
50
40
30
20
10
0
3.9
30.5
73.7
8
340 No Ab
340
340 LXSN
340 LUSN

Figure 7

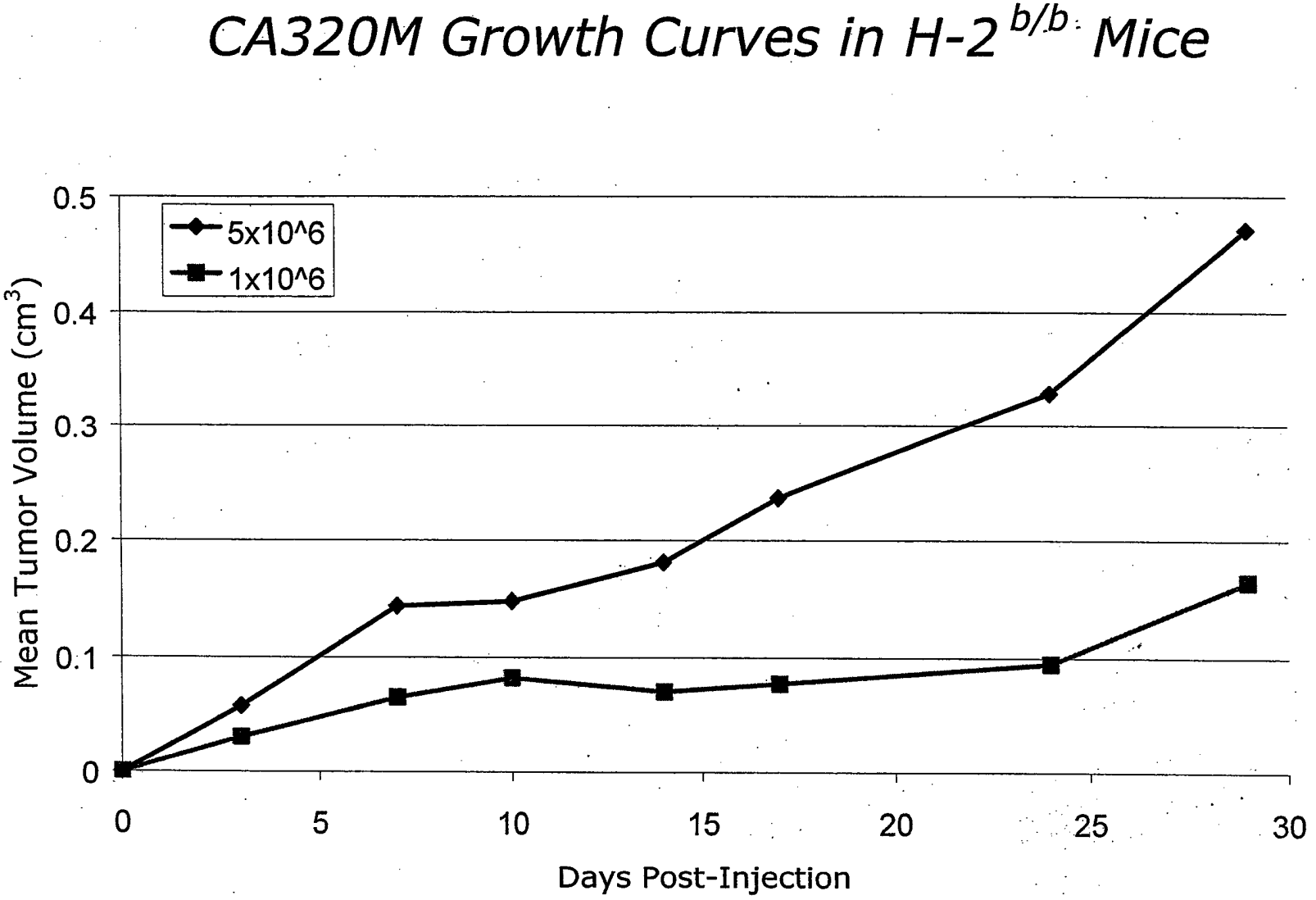

CA320M Growth Curves in H-2 b/b Mice
5x10^6
1x10^6
Mean Tumor Volume (cm3)
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
Days Post-Injection

Figure 8

48 h Post-transduction

3/12/03
MCF-7

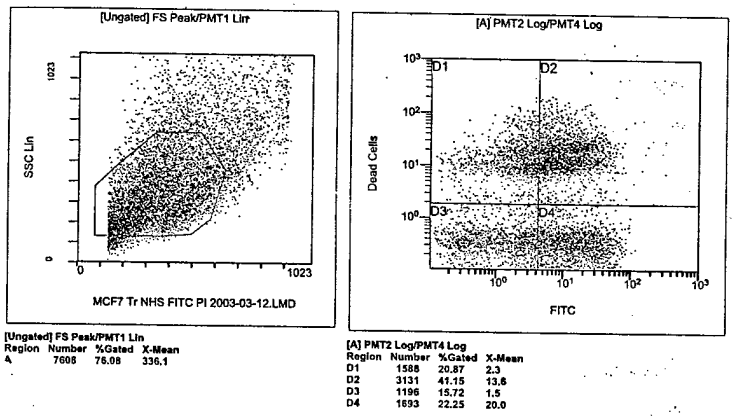
	Tube #1	Tube #2	Tube #3	Tube #4	Tube #5
Quadrant 1	20.87%	8.72%	0.00%	8.62%	4.76%
Quadrant 2	**41.15%**	12.98%	0.50%	0.07%	0.05%
Quadrant 3	15.72%	28.04%	98.62%	90.44%	94.74%
Quadrant 4	22.25%	50.26%	1.34%	0.87%	0.44%

3/12/03
BT-20

	Tube #1	Tube #2	Tube #3	Tube #4	Tube #5
Quadrant 1	3.40%	2.60%	0.00%	0.93%	0.89%
Quadrant 2	87.89%	16.63%	0.10%	0.28%	0.23%
Quadrant 3	3.65%	21.02%	99.99%	96.36%	97.74%
Quadrant 4	5.06%	59.76%	0.00%	2.43%	1.14%

3/12/03
T47D

	Tube #1	Tube #2	Tube #3	Tube #4	Tube #5
Quadrant 1	2.53%	1.94%	0.05%	1.09%	0.96%
Quadrant 2	87.38%	34.68%	0.00%	0.30%	0.11%
Quadrant 3	3.75%	5.52%	99.67%	98.11%	98.67%
Quadrant 4	6.34%	57.85%	0.27%	0.50%	0.26%

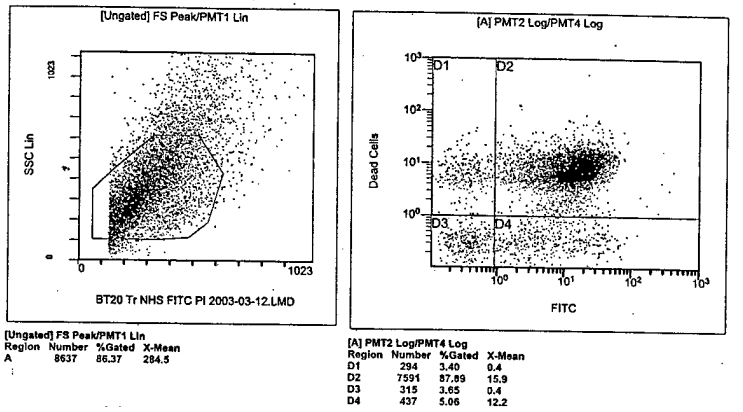


[Ungated] FS Peak/PMT1 Lin

Region	Number	%Gated	X-Mean
A	7608	76.08	336.1

[A] PMT2 Log/PMT4 Log

Region	Number	%Gated	X-Mean
D1	1588	20.87	2.3
D2	3131	41.15	13.6
D3	1196	15.72	1.5
D4	1693	22.25	20.0

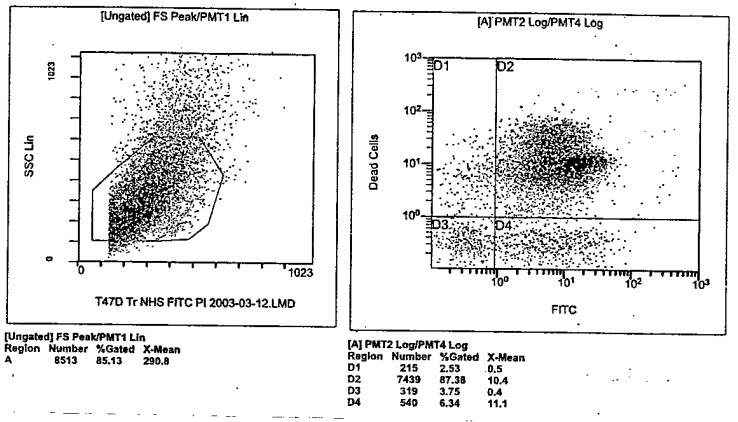


[Ungated] FS Peak/PMT1 Lin

Region	Number	%Gated	X-Mean
A	8637	86.37	284.5

[A] PMT2 Log/PMT4 Log

Region	Number	%Gated	X-Mean
D1	294	3.40	0.4
D2	7591	87.89	15.9
D3	315	3.65	0.4
D4	437	5.06	12.2



[Ungated] FS Peak/PMT1 Lin

Region	Number	%Gated	X-Mean
A	8513	85.13	290.8

[A] PMT2 Log/PMT4 Log

Region	Number	%Gated	X-Mean
D1	215	2.53	0.5
D2	7439	87.38	10.4
D3	319	3.75	0.4
D4	540	6.34	11.1

Figure 9

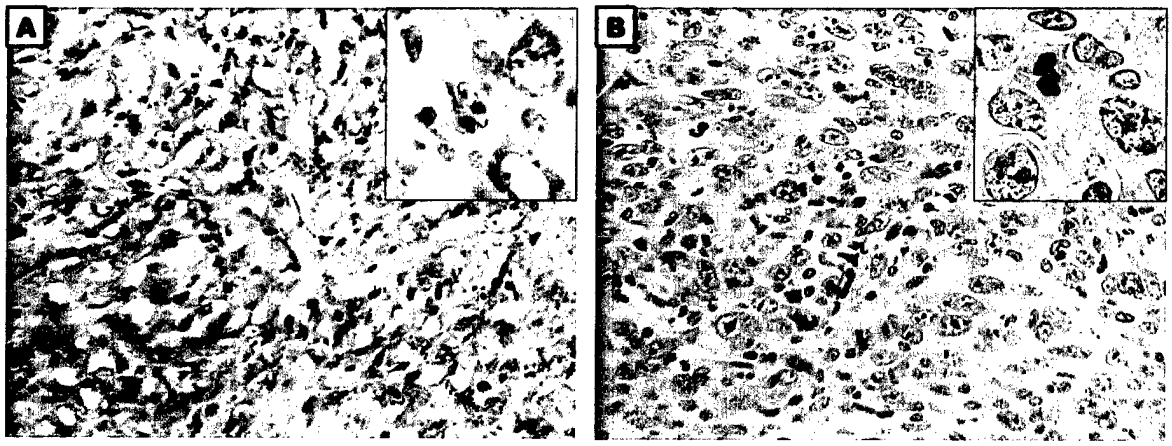


Figure 9. a. Frozen section of primary tumor, CA320M, derived from the gastrointestinal tract of a female mouse, 16x. Inset shows morphologies consistent with a sarcoma, 40x. b. Paraffin section of tumor arising from transplanted cultured CA320M cells injected s.c. in the abdomen of a female H-$2^{b/b}$ haplotype mouse, 16x. Inset shows mitotic figures at 40x magnification.

Figure 10

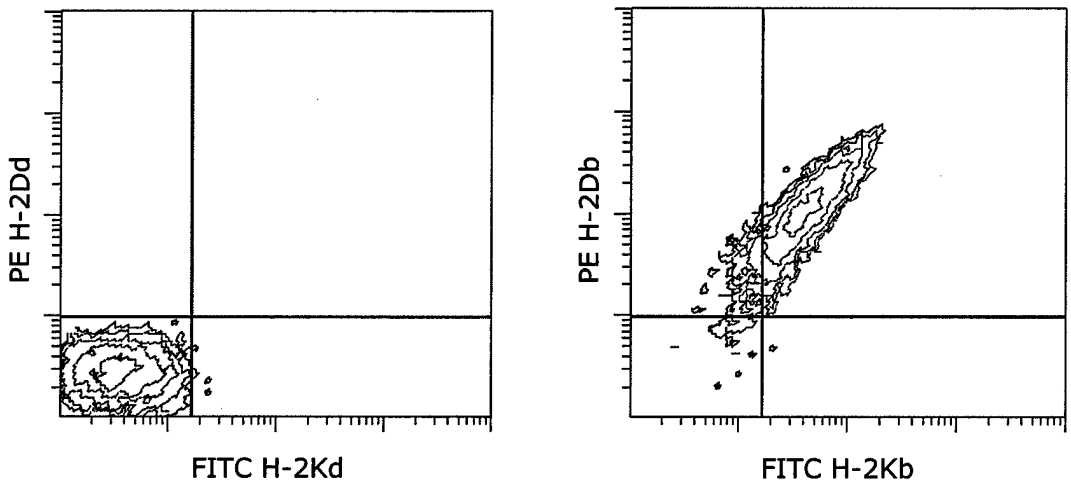


Figure 10. Flow cytometric analysis of CA320M haplotype. Antibodies against H-2Kb, H-2Kd, H-2Db, and H-2Dd were used on 10^6 cells. The histogram in the left panel shows dual labeled cells for the H-2d haplotype. The histogram in the right panel shows dual labeled cells for the H-2b haplotype. These data indicate that CA320M was derived from a homozygous H-$2^{b/b}$ mouse.

Figure 11

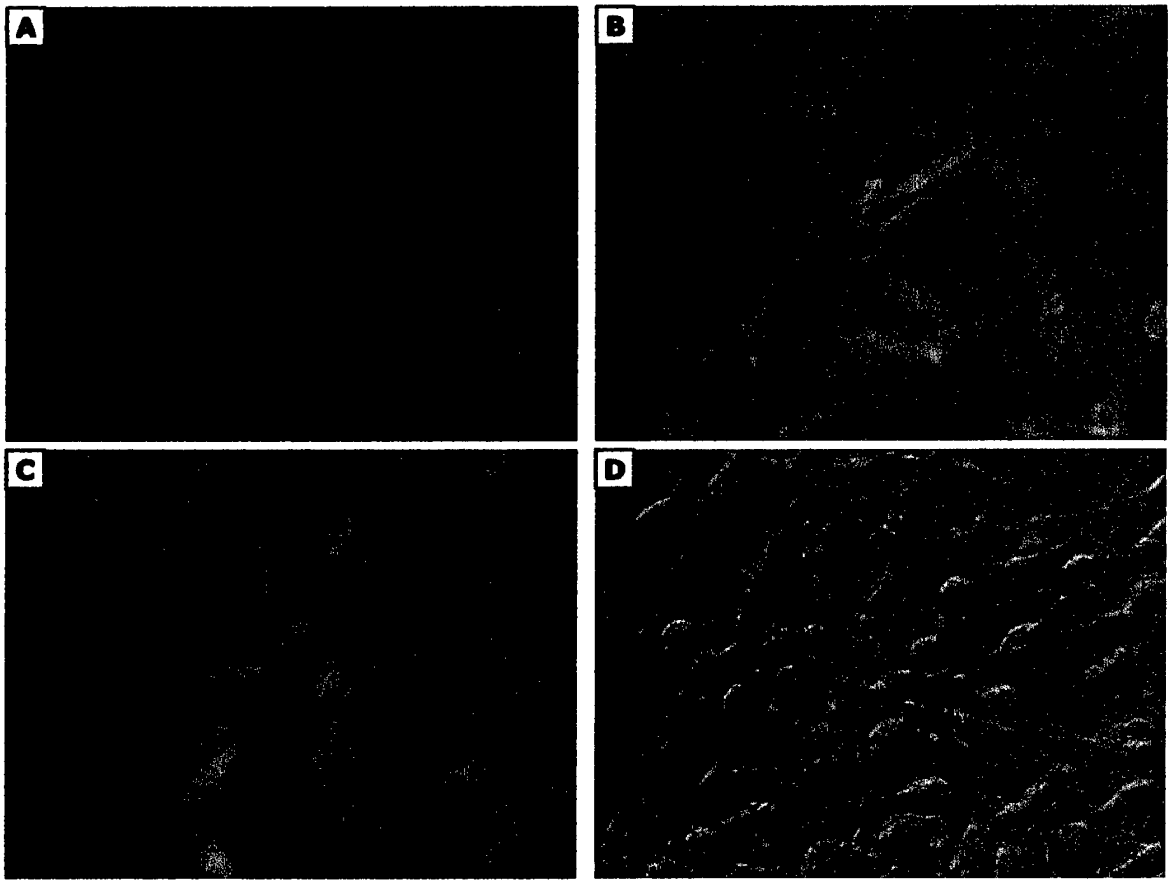


Figure 11. a. CA320M unmodified cells stained with FITC labeled IB_4 lectin specific for αgal epitopes. b. Cells transduced with HE7αgal1 for 8 hr showing the presence of cell surface αgal epitopes. c. Cells transduced with HE7EGFP as a control of efficiency of viral infection showing GFP fluorescence. d. Bright field of a representative culture of CA320M. 40x magnification.

Figure 12

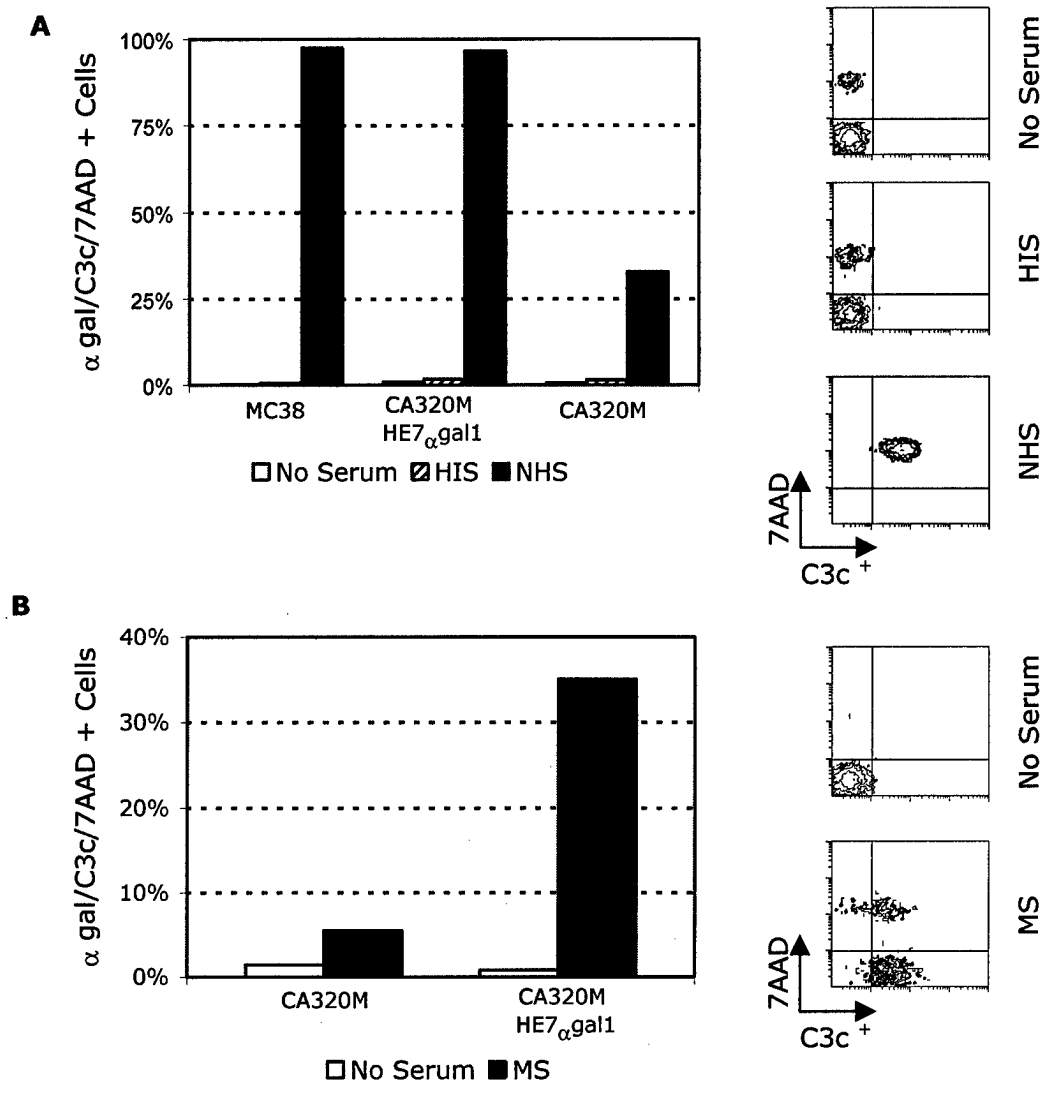


Figure 12. Serum killing analysis with a. normal human serum (NHS) (histograms on the right are of HE7αgal1 expressing cells gated on 7AAD and C3c staining) or b. serum collected from mice (MS) primed against the αgal epitope by 2 injections of RWB (histograms on the right are similar to above). Two hundred thousand cells were incubated with 50% serum for 1hr at 37°C and tri-labeled for αgal epitope, C3c complement, and cell death discrimination by 7AAD. Results are plotted as percent αgal positive C3c bound dead cells except CA320M which are only C3c/7AAD positive. HIS, Heat inactivated serum.

Figure 13

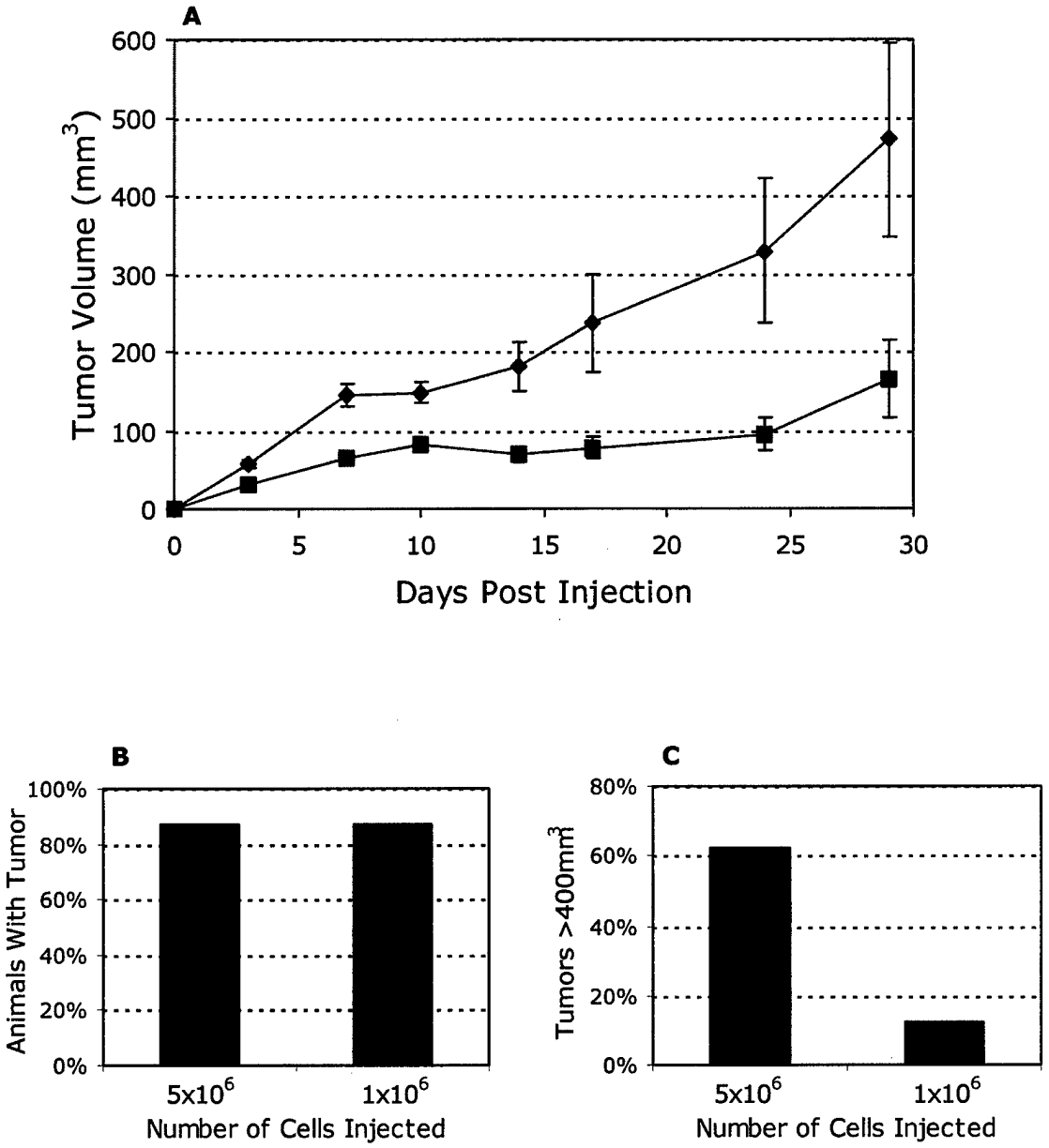


Figure 13. *In vivo* tumor growth kinetics of CA320M in αGT KO female H-$2^{b/b}$ mice. Mice (n = 8 per group) were injected subcutaneously with cells suspended in 100 µl HBSS. a. Mean (± SEM) tumor volume over time, [■] $1x10^6$ or [◆] $5x10^6$ cells per animal. Percentage of mice permitting tumor growth through day 30. c. Percentage of mice with lethal tumor volume of over 400 mm^3 by day 30.

Figure 14

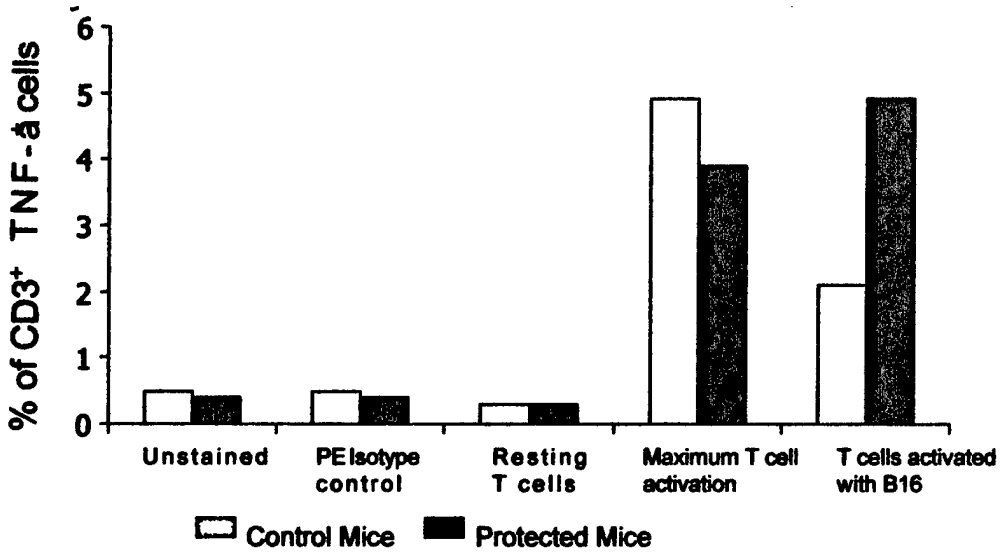


Fig. 14. Splenocytes were harvested from control mice mice and from mice protected after vaccination with aGal expressing B16 vaccines. T cells were cultured for 20 hs in presence or absence of stimulation with Brefeldin A to block secretion of cytokines. For maximum stimulation PMA/Ca++ Ionophoro were used. Cells were cultured with 10^5 irraditated B16 to measure specific recognition. After incubation cells were harvested, and double stained for CD3+ and intracytoplasmatic TNF-α. Detection was performed by FACS gating in CD3+ T cells.

Figure 15

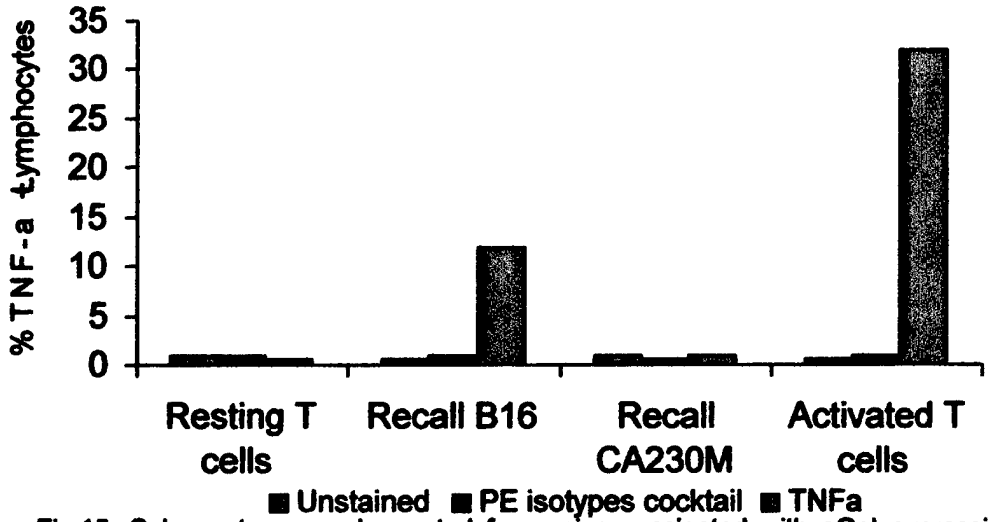


Fig.15: Splenocytes were harvested from mice vaccinated with aGal expressing vaccines. T cells were stimulated for 6 hs in presence or absence of stimulation with Brefeldin A to block secretion of cytokines. Cells were cultured with 10^5 irraditated B16 to measure specific recognition or with CA320M, a non-specific small intestine cell line with identical haplotype (b/b). After incubation cells were harvested and stained for intra cellular TNF-α. Detection was performed by FACS gating in lymphocytes in the Forward Scatter plot.

Eliciting Hyperacute Rejection of HSV α(1,3) Galactosyltransferase Transduced Tumors

Hellrung, DJ, Link, CJ, Human Gene Therapy Research Institute, Des Moines, IA.

ABSTRACT

Rejection of xenotransplants has been characterized by a major antigen, αgal, a product of the α(1,3) galactosyltransferase (αGT) gene. A golgi dependent enzyme, αGT, catalyzes the addition of a galactose from uridine diphosphate galactose (UDP-Gal) to the penultimate N-acetyl-lactosamine acceptor in an α (1,3) epitope (αgal) on glycoproteins and glycolipids. Moreover, mutations in this gene in humans, apes, and old world primates have resulted in an evolutionary divergence from all other mammals. Interestingly, in the former group, data suggests that 1% of circulating antibodies are directed against this carbohydrate epitope. It has been demonstrated that mimicking αgal epitopes are present on normal intestinal flora providing a possible explanation for such high anti-αgal titers. With this information we have sought to exploit this phenomenon in a gene therapy approach for the treatment of cancer. Therefore, human serum was collected from donors and frozen in aliquots to preserve complement factors. Fifty percent serum was mixed with MC38 αgal^+ murine colon carcinoma cells for 1 hour resulting in 98% killing. Based on this observation we used a herpes viral vector (HE7αgal1) system to deliver the murine αGT gene to various human tumor cell lines as well as a murine B16F10αgal^- melanoma cell line. After serum treatment as above, we noted a reduction up to 90% in cell viability against controls. In order to decipher the mechanism of tumor killing we repeated these experiments using complement inhibitor sCR1 or heat inactivated serum and noted restoration of viability to 95%.
Furthermore, *Griffonia simplicifolia* derived IB_4 FITC labeled isolectin specific for αgal epitopes stained MC38 cells and HE7αgal1 transduced tumors equally demonstrating cell surface expression of αgal in tranduced cells. Parental and mock transduced tumors were negative upon IB_4 isolectin staining. Based on these results we are further exploring the use of αGT in syngeneic αGT knock-out tumor models in an effort to understand the mechanism of tumor destruction *in vivo*. This work represents for the first time delivery of αGT via a herpes viral vector to tumors cells and subsequent direct tumor destruction *in vitro* using human serum.

Evidence for a protective immune stimulation from α (1,3) galactosyl epitopes in mice

Unfer, R.C., Hellrung, D., and Link C.J Human Gene Therapy Research Institute, John Stoddard Cancer Center, 1415 Woodland Ave. Suite 218 Des Moines, IA 50309

The α (1,3) galactosyl epitope (αgal) is recognized by the human immune system during complement-mediated hyperacute xenograft rejection. The future goal of *in vivo* delivery of the αgal gene into αgal-negative tumor cells, it's expression, and their subsequent killing is dependent upon demonstration of a protective immunity generated by exposure to αgal. Previous *in vitro* work by our Institute and others has demonstrated cytolysis of αgal transduced tumor cells by human serum. We designed two experiments to determine whether immune priming with αgal would provide any evidence for protection from tumor cell growth. In the first part of this study, C57BL/6 α (1,3) galactosyltransferase knockout mice (αgal-negative) immunized with αgal-rich rabbit RBC, and two non-immunized control groups of C57BL/6 α (1,3) KO mice, and wild-type C57BL/6 mice were implanted with dilutions of αgal-positive MC 38 colonic adenocarcinoma cells. Mice were monitored for tumor growth and morbidity. Results indicate a protective immunity was generated by prior exposure to the αgal epitope in immunized C57BL/6 α (1,3) KO mice signified by lower morbidity and increase survival. In the second part of this study, C57BL/6 α (1,3) galactosyltransferase KO mice were immunized with rabbit RBC, implanted with 10^5 cells of the αgal-negative B16.F10 tumor cell line and divided into three groups. Group A received only RBC and tumor cells, while groups B and C were implanted with 10^7 αgal-positive vector-producing cells (VPC). Group 3 mice were also treated with GCV (10μg/ml). Increased survival was observed among group C mice indicating a protective effect from both exposure to αgal-positive VPC and GCV. These data suggest that innate immunity in humans might be directed against α (1,3) galactosyltransferase expressing tumors.

Use of α(1,3) galactosyltransferase (αGT) as a Novel Suicide Gene for Cancer Gene Therapy.

Robert Unfer, Daniel Hellrung, Tatiana Seregina, Charles Link Jr. John Stoddard Cancer Research Institute, Iowa Methodist Medical Center, Des Moines, Iowa, Iowa State University, Ames, Iowa

A novel approach to cancer gene therapy uses the gene encoding α(1,3) galactosyl-transferase (αGT). αGT catalyzes the transfer of galactose to glycoproteins and glycolipids, creating the α(1,3) galactosyl epitope (αGal) on the cellular surface. The αGT enzyme is active in all lower mammalian species. During evolution, humans and higher apes lost functional activity of αGT, and all their cells are αGal negative. Innate immunity to αGal, stimulated by normal intestinal flora, comprises approximately 1% of circulating antibody in humans. Therefore, αGal is an important antigen involved in complement-mediated xenotransplant rejection, and αGT expression by cancer cells will provide a specific target for the human immune system. Previously we demonstrated that human anti-αGal antibodies contribute to the destruction of αGal^+ vector producer cells (VPC) of murine origin that are infused into ovarian cancer patients in an ongoing gene therapy Phase II clinical trial. We have also shown that human tumor cells lines transduced with a Herpes amplicon vector containing the αGT gene are destroyed by complement-mediated lysis following exposure to human serum. In our current work we have used αGT knockout mice as an *in vivo* model to demonstrate that an immune response to αGal can be protective when mice are challenged with murine tumor cells that are αGal^+. We immunized αGT knockout mice with rabbit red blood cells (RRBC) that are rich in αGal epitopes, and implanted αGal^+ murine colon carcinoma cells (MC38). Mice were monitored for tumor growth and morbidity. Results demonstrated that immunized αGT knockout mice exhibited slower tumor growth and survived (20/20 survived), while non-immunized control αGT knockout mice had more rapid tumor growth and a higher rate of mortality (12/20 survived). Antibody subtype analysis of serum from immunized mice showed a predominance of IgM and IgG3 heavy chain, and kappa light chain. The immune response to αGal in knockout mice was monitored following RRBC immunization using an ELISA specific for the αGal epitope. The immunized mice exhibited a classical primary and secondary immune response despite the fact that αGal is a carbohydrate antigen. We present here analysis of the immunoglobulin genes responsible for immunity to αGal in both mouse serum, and serum collected from ovarian cancer trial patients infused with αGal^+ VPC.

Generation of a recombinant adenovirus carrying the gene for α(1,3) galactosyl transferase, for use as a suicide gene in cancer immunotherapy.

Unfer, R.C.[1], Hellrung, D.[1,2], Higginbotham, J.N.[1], Vahanian, N.[1], Seth, P[1]., and Link C.J. Jr.[1,2]. Human Gene Therapy Research Institute[1], John Stoddard Cancer Center, Des Moines, IA, 50309 and Iowa State University[2], Ames, IA 50011

The α(1,3) galactosyl transferase enzyme (αGT) is expressed in most mammals but not in humans. αGT adds an α(1,3) linkage of galactose onto glycoproteins or glycolipids, and is a key determinant of tissue and organ xenograft rejection. The α(1,3) galactosyl epitope (αgal) is recognized by innate human immunity resulting in antibody dependent complement-mediated hyperacute xenograft rejection. In human cells, αGT is present as a pseudogene due to two single base deletions. Our previous in vitro work has demonstrated that human tumor cells transduced with a retroviral vector expressing the αGT gene are destroyed by anti-αgal antibody and complement. Additionally, using the MC38 (αgal+) colon adenocarcinoma cell line and αGT knockout mice that have been immunized with rabbit red blood cells (αgal+), we have also demonstrated in vivo that a strong immune response to agal will provide protection from tumor challenge. To achieve a high level of gene transfer, we have cloned the αGT gene into an adenovirus shuttle-vector, and used this to generate a recombinant adenovirus that carries the αGT gene. IGROV human ovarian cancer cells transduced with an MOI of 100 show high levels of expression of the agal epitope when stained with FITC-labeled IB4 lectin. This vector is being evaluated in a variety of human tumor cells, and an in vivo model is under development. The αGT gene may be an ideal suicide gene for immunotherapy of solid tumors using present gene delivery technology. The expression of this transgene by tumor cells and resulting modification of both cellular glycoproteins and glycolipids may provide multiple targets for the immune system, and should result in tumor destruction that is not dependent upon further pharmacological intervention.

A NOVEL THERAPY FOR BREAST CANCER BY INDUCING HYPERACUTE REJECTION

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This project presents an innovative approach to breast cancer gene therapy that exploits a naturally occurring physiologic process in humans. We propose that the alpha(1,3)galactosyltransferase [a(1,3)GT] gene in fact represents an ideal unconventional "suicide" gene to induce destruction of the tumor, because the mediators of cell death are inherent in the human immune system and not dependent on pharmacological intervention. Strong immunological barriers to xenotransplants from lower mammals into humans can destroy a transplanted solid organ within minutes, a process termed hyperacute rejection. The expression of the murine a(1,3)GT gene results in the cell surface expression of a(1,3)galactosyl epitopes (agal) on membrane glycoproteins. These epitopes are the major target of the human hyperacute rejection response that occurs when organs are transplanted from nonprimate donor species. a(1,3)GT is expressed in all mammals including Mus musculus, but not in old world primates, apes or humans. We employed a novel Herpes amplicon vector (HE7agal1) that efficiently infects human solid tumor cells at low multiplicity of infection and permits high-level transgene expression. The anti-tumor effectiveness of a(1,3)GT gene transfer has now been show in vitro in human breast tumor cell lines with rapid killing after normal human serum exposure by complement lysis. The a(1,3)GT knockout mouse serves as the only small animal model analogous to humans in which to study the immune response to the agal epitopes. a(1,3)GT KO mice immunized with rabbit RBC (agal+) produced high-titer anti-agal Ab responses in all mice. When agal immunized mice were challenged by injection with agal positive tumor cells, 70% to 100% of the mice survived up to 30 days. Control normal mice (agal+) challenged with the same tumor died uniformly before day 19 after challenge. Therefore, the presence of anti-agal Ab was highly protective. Next, a(1,3)GT KO mice were administered carcinogens to generate a murine breast cancer cell line (agal negative) for an in vivo tumor model. A murine breast cancer cell line (agal negative) was derived from these mice. In conclusion, our data show effective use of the murine a(1,3)GT gene as a therapeutic transgene to induce hyperacute rejection of breast cancer.

The U.S. Army Medical Research Materiel Command under DAMD17-00-1-0292 and the Susan G. Komen Breast Cancer Research Foundation supported this work.

Characterization of a gastrointestinal stromal tumor line derived from an α(1,3)galactosyltransferase knockout mouse and its evaluation as a potential gene therapy model

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ABSTRACT

Gastrointestinal malignancies represent 20% of newly diagnosed cases of cancer each year and 132,300 deaths from these tumors. Our group and others are exploring the use of α(1,3)galactose epitopes (αgal) to induce hyperacute rejection of tumors. Towards this goal, we developed and characterized a gastrointestinal stromal tumor line, CA320M, derived from αgal knockout (αGT KO) mice after chemical carcinogenesis. Mice were injected twice intraperitoneally with 9,10-dimethyl-1,2-benz-anthracene (DMBA), 3-methylcholanthrene (3MC), and 6α-methyl-17α-hydroxy-progesterone acetate (MHP) and followed for up to 24 months. Forty-four animals developed tumors of which 24 were successfully cultured including an adenocarcinoma of the breast, squamous cell carcinoma of the dermis and multiple fibrosarcomas. CA320M, a sarcoma of the small intestine, was obtained from the gastrointestinal tract of a female mouse and established in cell culture. Flow cytometric analysis demonstrated these cells to be homozygous for the H-$2^{b/b}$ haplotype with a normal diploid genome. CA320M cells replicated approximately every 12 hr. CA320M cells demonstrated no detectable cell surface αgal epitopes. Cells transduced with a Herpes Simplex Virus Type 1 (HSV-1) amplicon vector (HE7αgal1) carrying the murine αgal gene resulted in significant αgal epitope expression. CA320M cells, transduced with HE7αgal1, were incubated with pooled normal human serum and serum collected from αGT KO C57/BL6 mice immunized with αgal positive rabbit whole blood (RWB). Flow cytometric analysis of serum exposed αgal expressing cells demonstrated up to 97% anti-αgal antibody mediated complement lysis within 1 hr. The αGT KO mice were bred to obtain an H-$2^{b/b}$ haplotype population. Of the 24 cultured lines, CA320M was the only tumor model to survive reproducibly in naive animals. Female H-$2^{b/b}$ mice were challenged with subcutaneous injections of CA320M cells. Cells ($5x10^6$) injected subcutaneously produced approximately 500 mm^3 tumors within 30 days. Histopathological examination of the resulting tumors demonstrated morphologies consistent with a poorly differentiated sarcoma. The αGT KO tumor model, CA320M, may allow investigators to further explore the biology of αgal vaccine strategies against gastrointestinal malignancies.

Adenoviral Gene Delivery of α(1,3)Galactosyltransferase to Breast Cancer Cells: Tumor Cell Killing by Innate Immunity

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Cancer gene therapy offers an additional method of treatment for cancer patients who have relied on traditional cancer therapies. Progress is continuing in the development of protocols that effectively destroy tumor cells, and can either stand alone, or can work together with other methodologies to control tumor growth and prolong survival. Hyperacute rejection of xenotransplants is caused by innate human immunity to the major xenoepitope (αGal) found on the surface of all lower mammalian cells. We have demonstrated that when the murine α(1,3)Galactosyltransferase gene (αGT) is transduced into human tumor cells, surface membrane glycoproteins and glycolipids are glycosylated to express the αGal epitope. Polyclonal anti-αGal antibodies in immune complexes induce activation of complement, and a reaction similar to complement-mediated hyperacute xenograft rejection occurs. αGT has the potential to be an effective suicide gene that can induce destruction of human tumor cells without additional pharmacological agents. We cloned the murine αGT gene into a replication-defective adenoviral vector, and are evaluating the ability of this gene to induce tumor cell death when transduced cells are exposed to normal human serum (NHS). Three human breast cancer cell lines MCF-7 (Breast adenocarcinoma); T47D (Breast ductal carcinoma); and BT-20 (Breast tumor) were transduced with Ad-αGT (MOI = 100) and analyzed for αGal expression by binding of FITC-labeled isolectin B4 (IB4) that is specific for αGal epitopes. A cell killing assay using pooled NHS was performed on transduced cells at 24 h, 48 h, and 96 h post transduction. In flow cytometry of 48 h post-infection T47D and BT-20 cells, there was high transduction and expression (with 78% and 80% respectively), while 52% of MCF-7 cells expressed αGal epitopes. Incubation of transduced cells with 50% NHS resulted in significant cell killing of T47D and BT-20 cells observed by propidium iodide staining (72% and 70% respectively) that peaked at 48 h. MCF-7 cells were also killed in the presence of serum (32%). Therefore, Ad-αGT delivery of the αGT gene leads directly to tumor destruction by NHS in a cell independent manner, and further development of this methodology may lead to an effective cancer gene therapy.

Cancer Gene Therapy: Adenoviral Delivery of the Suicide Gene αGT Results in Tumor Cell Killing by Innate Immunity

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Gene therapy protocols for cancer have been available for clinical testing for more than a decade now. The optimism for this new technology can be restored with new therapeutic genes that offer novel and effective methods to kill tumor cells. Science has known that hyperacute rejection of xenotransplants in humans is mediated by innate immunity to the major xenoepitope (αGal) found on the surface of all lower mammalian cells. The α(1,3)Galactosyltransferase gene (αGT) catalyzes the formation of the αGal epitope, and has the potential to be an effective suicide gene that can induce destruction of human tumor cells without additional pharmacological agents. We have shown that anti-αGal antibodies form immune complexes with αGT transduced cells and induce activation of complement that results in cell death. In order to improve gene delivery, we have cloned the murine αGT gene into a replication defective adenoviral vector (Ad-αGT). Transduced human SKOV-3 (ovarian), DU145 (prostate), BT-20 (breast), and A549 (lung) cancer cells (MOI = 100) were analyzed for αGal expression by binding of FITC-labeled isolectin B4 (IB4) which is specific for αGal. The *in vitro* ability of αGT expression to induce tumor cell death when transduced cells are incubated with 50% pooled normal human serum (NHS) was evaluated in a serum-killing assay. Flow cytometry was used 48 h post-transduction to measure αGal expression and tumor cell killing. All tumor cell lines with the exception of A549 were transduced with a high percentage of cells exhibiting αGal expression (89%, 93%, and 96% respectively). A549 cells exhibited 60% expression of αGal. Incubation of transduced cells with pooled NHS and staining with propidium iodide demonstrated significant killing of SKOV-3, DU145, and BT-20 (91%, 85%, and 80% respectively). Transduced A549 cells were also killed in the presence of serum (34%). Therefore, delivery and expression of the αGT gene directly leads to tumor destruction by NHS in a cell independent manner.

Characterization of a gastrointestinal stromal tumor line derived from an α(1,3)galactosyltransferase knockout mouse and its evaluation as a potential gene therapy model

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Key Words: α(1,3)galactosyltransferase, chemical carcinogenesis, tumor model, small intestine tumor, gene therapy, xenotransplantation

Abbreviations:

HSV-1: Herpes Simplex Virus Type 1
RWB: rabbit whole blood
FITC: fluorescein isothiocyanate
DMBA: 9,10-dimethyl-1,2-benz-anthracene
α(1,3)GT: α(1,3)galactosyltransferase gene
αgal: α(1,3)galactosyltransferase epitope
αGT KO: α(1,3)galactosyltransferase knockout
3-MC: 3-methylcholanthrene
IB_4: isolectin B4
PE: phycoerythrin

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Running Title: A new Galα(1,3)Gal negative tumor line

OVERVIEW SUMMARY

Gastrointestinal malignancies remain a significant contributor to the overall death rate due to cancer. Immunotherapies are showing promise in potentially treating these devastating diseases. We are investigating the use of Galα(1→3)Gal (αgal) epitopes to induce hyperacute rejection of tumor vaccines in order to potentiate an immune response against tumor antigens. However, currently there are no published reports of a murine gastrointestinal αgal negative tumor model in which to study this approach. Therefore we have developed and characterized a gastrointestinal stromal tumor of the small intestine, CA320M. Moreover, using an HSV-1 amplicon delivery vehicle the αgal gene was transferred to these cells resulting in significant αgal epitope expression. Using normal human serum which contain pre-existing anti-αgal antibody and mouse serum derived from αGT KO mice primed against the αgal epitope by repeated injection of rabbit whole blood cells, we demonstrated that αgal positive CA320M cells are susceptible to complement mediated hyperacute rejection. These data indicate that this model may be useful in studying αgal modified tumor vaccines *in vivo*. Moreover, CA320M may be used to study the biology of gastrointestinal tumors utilizing an αgal knockout mouse making this tumor a valuable addition to the currently existing murine tumors models.

(LaTemple *et al.*, 1999). In those experiments 30% of the mice survived lethal tumor challenge. However, the B16.BL6/αgal model may not predict the outcome of other tumor types including gastrointestinal malignancies. Currently no other αGT KO tumor models have been reported for other tumor types. Therefore, in order to investigate tumor therapies potentially relevant for gastrointestinal cancers an additional tumor model is needed. Towards that end, αGT KO mice were administered 9,10-dimethyl-1,2-benzanthracene, 3-methylcholanthrene, and 6α-methyl-17α-hydroxy-progesterone acetate to induce tumors. A population of chemically treated mice were followed for up to two years. Here we characterize a new gastrointestinal stromal tumor from the small intestine, CA320M, and show its usefulness as a tumor model to study αgal vaccine tumor therapies.

Histopathology of Isolated Tumors

Fixed tumors were dehydrated with 25% sucrose dissolved in 1x PBS, for 3 days. Thin sections were obtained by snap freezing with 1x PBS to a stage and sectioning at 5-10 μm on a Leica CM1850 cryostat. Sections were allowed to air dry overnight and then stained with hematoxylin/eosin according to standard procedures (Ausubel *et al.*, 1995). Paraffin embedded sections of transplanted CA320M tumors were obtained by standard techniques (Iowa State University Immunohistochemistry Services, Ames, IA). Sections were stained with hematoxylin/eosin and probed for actin (smooth muscle specific and muscle specific) and myoglobin.

Haplotyping of Parental Mice and CA320M Tumor Line

Phycoerythrin (PE) labeled Anti-H-2D^b, fluorescein isothiocyanate (FITC) labeled anti-H-2K^b, PE labeled anti-H-2D^d and FITC labeled anti-H-2K^d antibodies with isotype controls (BD Pharmingen, San Diego CA) were used to haplotype both the parental mice and the CA320M tumor. Blood was drawn from the saphenous vein of up to 300 mice and each sample was washed in 1x HBSS and approximately 10^6 cells were stained with 1 μg of each of the antibodies above in a 96-well plate (Corning, Corning NY). Labeled leukocytes were positively identified under fluorescent microscopy. Haplotype matched mice were bred and used for the remainder of this work. Approximately 10^6 CA320M cells were labeled with each of the antibodies above and analyzed by flow cytometry (Beckman Coulter Epics Altra).

Determination of Cell Cycle and Proliferation Rate in Normal and Serum Starved Conditions

Adherent CA320M or B16.BL6.2 cells were harvested from tissue culture flasks by incubating with 0.4% trypsin 0.05 mM EDTA (Invitrogen-Life Technologies, Inc.) for 2 min at 37°C. Cells were then seeded in 8 flasks and cultured in D10. Twenty-four hours later media of 4 flasks was replaced with DMEM supplemented with 0.1% FBS (D0.1) and cultured for 12, 24, 36, and 48 hr. Cells from 0 hr and each subsequent time point were harvested as above and analyzed for cell cycle by flow cytometry after staining with Coulter DNA-Prep Reagents Kit (Coulter Co, Miami, FL). Cell ploidy was determined by using the 0 hr time point. Proliferation rate was determined by culturing cells stained with carboxy-fluorescein diacetate, succinimidyl ester (CFSE) in similar conditions as above for 0, 24, and 48 hr time points. Cells ($1x10^6$) for each time-point were incubated with 5 μm CFSE at 37°C for 2 min and the reaction was terminated by the addition of iced D10. Cells were washed in HBSS and plated in either D10 or D0.1 for the duration

Dickinson Labware, Franklin, NJ) were coated with 50 µl of 5 µg/ml αgal-BSA conjugate (Vector labs) in carbonate buffer (pH 9.5) overnight at 37°C in a humidified chamber. Plates were blocked with 150 µl of 1% BSA Fraction V (Sigma) in carbonate buffer for 2 hr at 37°C in humidified chamber. Sera was serially diluted in wash buffer (1x PBS/0.05% Tween 20 pH 7.2) to a final volume of 50 µl/well and incubated at room temperature for 2 hr. Wells were washed 5x in wash buffer followed by incubation with 50 µl/well of 1:3000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Dako A/S, Denmark) in wash buffer for 1 hr at room temperature. Wells were washed 5x followed by addition of 100 µl/well 3,3',5,5'-tetramethylbenzidine (Sigma) liquid substrate. The reaction was allowed to develop for 30 min at room temperature where upon it was terminated by the addition of 100 µl/well of 1 M H_2SO_4 and the results read at 450 nm.

CA320M cells were transduced with 3 MOI of either HE7αgal1 or HE7EGFP1 virus and incubated 8 hr at 37°C. Pooled sera from normal human donors or primed mice (described above) were incubated at a concentration of 50% with $2x10^5$ cells for 1 hr at 37°C. Cells were washed in HBSS and tri-labeled with 1 µg/ml biotinylated anti-αgal IB_4 (Vector Labs) 1:2000 dilution anti-C3c FITC labeled IgG (Dako A/S) and 1 µg/10^6 cells of 7-aminoactinomycin D (7-AAD, Sigma) in 1% BSA and HBSS for 30 min room temperature. The biotinylated IB_4 was detected with 0.5 µg/ml streptavidin conjugated PE. Cells were washed in HBSS and analyzed by flow cytometry.

Tumor Growth Kinetics In Vivo

Cultured cells were disassociated, pelleted, and resuspended in 10 ml HBSS. Cells were washed as above and pellets were resuspended in 5 ml HBSS and counted by trypan blue exclusion. Each of eight 8-14 wk old female haplotype matched animals received either $1x10^6$ or $5x10^6$ CA320M cells diluted in 100 µl HBSS via subcutaneous injection on the shaved ventral surface of the abdomen and followed for 60 days. Tumor volumes were calculated by multiplying the length, width, and height of each measured tumor and plotted over time.

Haplotyping of Parental Mice and CA320M Tumor Line

Tumors were developed in outbred αGT KO mice. The genetic background of these mice were determined to be a cross between C57/BL6, DBA/2 and 129SV (Thall *et al.*, 1995). Possible haplotypes were, therefore, limited to homozygous H-$2^{b/b}$ or H-$2^{d/d}$ or heterozygous H-$2^{b/d}$ (Kuby, 1994). Antibodies specific for these haplotypes were used to identify the haplotype of both the parental mice and the CA320M tumor. Three hundred mice were typed and matching haplotypes were bred for further use. Flow cytometry using these same antibodies demonstrated that CA320M was homozygous H-$2^{b/b}$ (Fig. 2). Mice of the same haplotype were bred resulting in two colonies representing both H-$2^{b/b}$ and H-$2^{d/d}$ haplotypes. Because CA320M was isolated from a female mouse and determined to be H-$2^{b/b}$ haplotype, all subsequent *in vivo* experiments were conducted in female H-$2^{b/b}$ αGT KO mice.

Determination of Cell Cycle and Proliferation Rate in Normal and Serum Starved Conditions

To determine the "putative tumorigenic" potential of the diploid CA320M tumor line, cell cycle and proliferation rate analysis was performed following culture in normal serum conditions and serum starved conditions. As a reference point B16.BL6 was used as it is a well characterized murine αgal negative tumor line. Cells were cultured for up to 48 hr in normal (D10) or serum starved (D0.1) conditions. The percent CA320M cells in S phase began to decline at 36 hr and by 48 hr reached 18.4% (Fig 3a). By contrast percent cells in G1 phase steadily increased to 80% indicating cells were arresting in G1 (Fig. 3b). Similarly, B16.BL6 demonstrated growth arrest after 48 hr culture in D0.1 (Fig. 3c, 3d). Proliferation rate was determined by incorporation of the die CFSE into cells cultured with D10 or D0.1 for up to 48 hr (Fig. 3e). When grown in D10 cells double every 12 hr resulting in approximately 3-4 doublings during a 48 hr period. Cells cultured in serum starved conditions followed a similar pattern for the first 24 hr and over 48 hr, 3 doublings were observed (Fig. 3f). These data correlate well with the variations in cell cycle noted above in murine tumor cells cultured in similar conditions.

HSV-1 Gene Transfer and Analysis of αgal Expression

CA320M cells induced in a αGT KO mouse failed to bind IB_4 isolectin (Fig. 5a), that specifically detects αgal epitopes, verifying that this murine tumor is, as expected, devoid of functional αGT enzyme and, therefore, αgal epitopes. CA320M cells were susceptible to infection by an HSV-1 based vector and expressed αgal epitopes after transduction with HE7αgal1. HE7EGFP was used as a control for transduction efficiency. Eight hr

DISCUSSION

Gastrointestinal malignancies represent a significant contributor to the overall death rates due to cancer each year. In 2002 alone, 132,300 people died of these cancer's representing 24% of the overall fatalities from cancer (Jemal *et al.*, 2002). Treatments using tumor vaccines are being explored in an attempt to induce an immune response to native tumor antigens. One of the approaches consists of inducing hyperacute rejection of tumor vaccines using α(1,3)galactosyltransferase gene therapy. With this approach it may be possible to use αgal modified tumor vaccines to education the immune system to recognize native tumor antigens. Alpha (1,3)galactosyltransferase (αGT) modifies glycoproteins and glycolipids to express the αgal epitope, Gal α(1→3)Gal (Larsen *et al.*, 1989; Joziasse *et al.*, 1992). Humans fail to express αgal epitopes due to mutations in both alleles of the αgal gene but have high titer antibody directed against this epitope (Galili *et al.*, 1984; Galili *et al.*, 1988a; Galili and Swanson, 1991; Joziasse *et al.*, 1991; Hamadeh *et al.*, 1992). The presence of anti-αgal antibody primes the immune system to respond to αgal epitopes resulting in complement mediated lysis of tissue expressing these epitopes. It has been shown that human and murine tumor cells engineered to express the αgal epitope are sensitized to αgal mediated complement lysis when incubated for 1 hr with 50% normal human serum. (Link *et al.*, 1998; Jager *et al.*, 1999; LaTemple *et al.*, 1999; Unfer *et al.*, 2003). However, few small animal tumor models exist to study this vaccine approach, particularly with respect to gastrointestinal malignancies *in vivo*.

LaTemple et al., used the B16.BL6 line as a model for αgal mediated tumor therapy of melanoma (LaTemple *et al.*, 1999). In their experiments, animals where immunized with RWB, vaccinated with either αgal expressing B16.BL6 or parental B16.BL6, followed fourteen days later by challenge with parental B16.BL6 tumor cells. They demonstrated that approximately 30% of the animals survived lethal challenge and raised the question as to whether cell mediated immunity against native tumor antigens had developed in those animals. One of the drawbacks of using the B16 model is that it has been reported to be highly metastatic (Hart, 1979) and variable in its immunogenic properties (Steele *et al.*, 1980; Nanni *et al.*, 1983; Heath and Boyle, 1985; Stackpole *et al.*, 1987). Moreover, in that study the animals used were outbred resulting in a variable background of H-2d and H-2b haplotypes and B16.BL6 was derived from C57/BL6 and therefore is H-2b haplotype. Therefore, the predictive capacity of B16.BL6/αgal model in those studies may be suboptimal for other tumor types including gastrointestinal malignancies.

Studies by Unfer *et al.*, using the murine αgal positive colon carcinoma, MC38, have shown protection from lethal tumor challenge in haplotype matched αGT KO mice (Unfer *et al.*, 2003). Mice were immunized against the αgal epitope using RWB followed by lethal challenge with MC38 cells. In that study, 100% of the animals were protected as compared to controls. However, since the MC38 model endogenously expresses αgal, the

gene therapy relies on gene delivery systems, we sought to determine if CA320M was susceptible to infection by Herpes Simplex Virus Type 1 (HSV-1) amplicon vectors. Among the many gene transfer techniques available, HSV-1 amplicons are attractive because they can carry large fragments of DNA, have greater than 90% tropism, infect nondividing cells, and can be prepared in high titers (Lim *et al.*, 1996; Wang *et al.*, 1997). Therefore, we used an amplicon vector system to deliver the murine α(1,3)GT gene or EGFP. The virus successfully infected CA320M as detected by EGFP fluorescence within 8 hr (Fig 4c). Also, αGT expression resulted in the modification of cell surface molecules to present αgal epitopes as detected by FITC labeled IB_4 lectin binding (Fig. 4b). These results indicate that CA320M is capable of being infected by HSV-1 vectors and cellular mutations have not inhibited the functional expression of αGT enzyme.

Previous investigators have demonstrated lysis of αgal expressing human tumor cells such as A375 melanoma (Link *et al.*, 1998), ALL-1 pre-B leukemia (LaTemple *et al.*, 1996) as well as murine tumor cells such MC38 colon carcinoma and B16.BL6 (LaTemple *et al.*, 1999; Unfer *et al.*, 2003). To evaluate the efficiency of hyperacute rejection of αgal expressing CA320M, cells were infected with HE7αgal1 for 8 hr followed by incubation with 50% normal human serum (NHS) or serum collected from mice primed with RWB (MS). Ninety-seven percent of αgal expressing CA320M cells bound anti-C3c antibody and stained with 7AAD demonstrating cell death when incubated with NHS (Fig. 5a). By comparison, 98% of MC38 cells, derived from wild-type C57/Bl6 mice that are naturally αgal positive, were killed. To determine if this was a response to a xenoantigen independent of αgal epitopes or a component of the viral toxicity, CA320M cells alone or infected with HE7EGFP, expressing the enhanced green fluorescent protein, were incubated with NHS as above. Thirty-three percent of parental CA320M compared to 40% of EGFP expressing cells were killed demonstrating that NHS can recognize other epitopes on CA320M independent of the virus. However, αgal expression increased the killing of these cells 3-fold indicating that the increase in cell lysis was due to the expression of αgal epitopes. Moreover since complement is easily destroyed by heat, these cells were incubated with heat inactivated serum and only 2% of the cells were killed.

Incubation with MS killed 35% of αgal expressing cells (Fig. 5b). As compared to lysis of only 6% of the cells expressing EGFP and 5.5% of the parental CA320M. These data correlate well with NHS results by showing up to a 7-fold increase in αgal specific killing over controls. Moreover, these results suggest that the CA320M tumor model would be well suited for studying hyperacute rejection as a cancer therapy strategy *in vivo* as anti-αgal mouse serum is capable of killing these cells.

Finally, CA320M growth kinetics was studied to establish parameters to use for *in vivo* tumor vaccine studies. One and five million cells were injected subcutaneously into 8-14 wk old mice. Tumors grew in 90% of the animals reaching a mean tumor volume of 164 mm^3 and 471 mm^3, respectively, by day 30. In the latter group, 63% of the animals

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Figure 5. Serum killing analysis with a. normal human serum (NHS) (histograms on the right are of HE7αgal1 expressing cells gated on 7AAD and C3c staining) or b. serum collected from mice (MS) primed against the αgal epitope by 2 injections of RWB (histograms on the right are similar to above). Two hundred thousand cells were incubated with 50% serum for 1hr at 37°C and tri-labeled for αgal epitope, C3c complement, and cell death discrimination by 7AAD. Results are plotted as percent αgal positive C3c bound dead cells except CA320M which are only C3c/7AAD positive. HIS, Heat inactivated serum.

Figure 6. *In vivo* tumor growth kinetics of CA320M in αGT KO female H-$2^{b/b}$ mice. Mice (n = 8 per group) were injected subcutaneously with cells suspended in 100 µl HBSS. a. Mean (± SEM) tumor volume over time, [■] $1x10^6$ or [◆] $5x10^6$ cells per animal. Percentage of mice permitting tumor growth through day 30. c. Percentage of mice with lethal tumor volume of over 400 mm^3 by day 30.

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Immunity to the α(1,3)Galactosyl Epitope Provides Protection in Mice Challenged with Colon Cancer Cells Expressing α(1,3)Galactosyl-transferase: A Novel Suicide Gene for Cancer Gene Therapy[1]

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ABSTRACT

Human immunity to α(1,3)Galactosyl epitopes (αGal) may provide the means for a successful cancer gene therapy that uses the immune system to identify and to destroy tumor cells expressing the suicide gene α(1,3)Galactosyltransferase (αGT). Innate antibody specific for cell surface αGal constitutes a high percentage of circulating IgG and IgM immunoglobulins in humans and is the basis for complement-mediated hyperacute xenograft rejection and antibody-dependent cell-mediated cytotoxicity. In humans, the gene for αGT is mutated, and cells do not express the αGal moiety. We hypothesized that human tumor cells induced to express the αGal epitope would be killed by the hosts' innate immunity. Previous *in vitro* work by our group has demonstrated complement-mediated lysis of αGal-transduced human tumor cells in culture by human serum. To induce antibodies to αGal in this *in vivo* study, αGT knockout mice were used to determine whether immunization with αGal could provide protection from challenge with αGal-expressing murine MC38 colon cancer cells. Knockout mice were immunized either a single time, or twice, with rabbit RBC. Antibody titers to αGal measured by indirect ELISA were significantly higher in mice immunized twice and approached the titers observed in human serum. Anti-αGal antibodies were predominantly of the IgG1 and IgG3 subtype. Immunized knockout mice were challenged i.p. with varying doses of αGal$^+$ MC38 colon carcinoma cells. Nonimmunized control groups consisting of αGT knockout mice, and wild-type C57BL/6 mice were challenged as well with MC38 cells. Immunized mice survived and exhibited slower tumor development in comparison to nonimmunized knockout and control mice. This study demonstrates, *in vivo*, the protective benefit of an immune response to the αGal epitope. Our results provide a basis to pursue additional development of this cancer gene therapy strategy.

INTRODUCTION

Cancer gene therapy offers a potential replacement or augmentation of traditional cancer treatments, which use invasive or toxic protocols. Suicide genes that encode an enzyme that activates a prodrug into a toxic molecule, or genes that induce apoptosis, have been or are currently being tested in clinical trials for their efficacy in cancer therapy (1–3). Gene therapy vaccine technology is under development for several malignancies. Melanoma has been the favored target because it is a very immunogenic tumor. However, the more common and important forms of cancer have minimal or no immunogenicity. We are developing a novel approach to cancer gene therapy that uses a patient's innate immunity against xenoantigens to identify and destroy tumor cells. The key aspect of this work is to determine whether colon cancer cells that express a xenoantigen can be rejected by an immune response to the antigen. Colon cancer is the third most common form of cancer and third leading cause of death (4) and provides a good target for a successful cancer gene therapy.

Humans possess specific humoral immunity to αGal,[3] a major xenotransplant antigen. Although human cells do not carry a functional enzyme for the expression of αGal epitopes because of a 2-base frameshift gene mutation (5), there is evidence that suggests that high titer natural antibody to αGal is produced in humans because of continuous antigenic stimulation by gastrointestinal bacteria (5–7). Clonal B-cell analyses estimated that ~1% of circulating B cells produce anti-αGal antibody (8). The αGT catalyzes the transfer of galactose from UDP galactose to the *N*-acetyl-lactosamine acceptors on carbohydrate side chains of glycoproteins and glycolipids to create the αGal moiety. The anti-αGal immune response is responsible for initiating hyperacute rejection of vascularized xenotransplants, a severe immunological reaction observed in primates. When αGal and specific antibody form immune complexes, complement is activated via the classical pathway (9–13).

Our interest in αGal-mediated destruction of tumor cells was inspired by studies describing lysis of murine retroviral VPCs after exposure to human peritoneal fluid. VPCs have been used for *in vivo* gene delivery in several cancer gene therapy studies (14, 15). Our laboratory and others have demonstrated that antibody and complement in human serum binds αGal within 30 min of exposure and induces complement-mediated lysis of VPCs and the viral vectors they produce (16–20). Additionally, Collins *et al.* (21) showed that human fibroblast cells expressing porcine αGT were destroyed by antibody and complement. To test whether this gene could be used to induce destruction of tumor cells, a truncated version of the murine αGT was cloned into a retroviral vector backbone and used to transduce human A375 melanoma cells (22). During *in vitro* experiments, >90% of transduced A375 cells expressing αGal were killed after exposure to human serum. αGal-expressing A375 cells were treated for 30 min with human serum and then injected *in vivo* into athymic nude mice. All experimental mice remained tumor free, whereas control groups developed tumors (22). Lysis of αGal-expressing murine cells by human serum can be blocked by the addition of complement inhibitors (heparin, enoxaparin) or soluble complement receptor 1 (20). These data demonstrate the key role that complement has in destruction of targets expressing the αGal xenoantigen.

Transgenic knockout mice that lack the αGT gene (αGT KO) have been produced (23, 24) and provide an ideal small animal model to study the *in vivo* immune response against αGal epitopes. While not expressing detectable cell surface αGal epitopes, these mice can produce low detectable titers of natural anti-αGal, possibly from bacterial stimulation (25, 26). Immunization with RRBCs results in the production of anti-αGal antibody with titers and specificity similar to those observed in humans (27). In this report we present *in vivo* data that shows clear protective benefits of an anti-αGal immune response, when RRBC-immunized αGT KO mice are challenged with αGal$^+$ tumor cells. These findings have implications for generation of a system to deliver the αGal suicide gene to human tumor cells, and

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[3] The abbreviations used are: αGal, α(1,3)Galactosyl epitope; αGT, α(1,3)Galactosyltransferase enzyme; αGT KO, α(1,3)Galactosyltransferase knockout; VPC, vector producer cell; RRBC, rabbit RBC; IB4, isolectin B4; PI, propidium iodide.

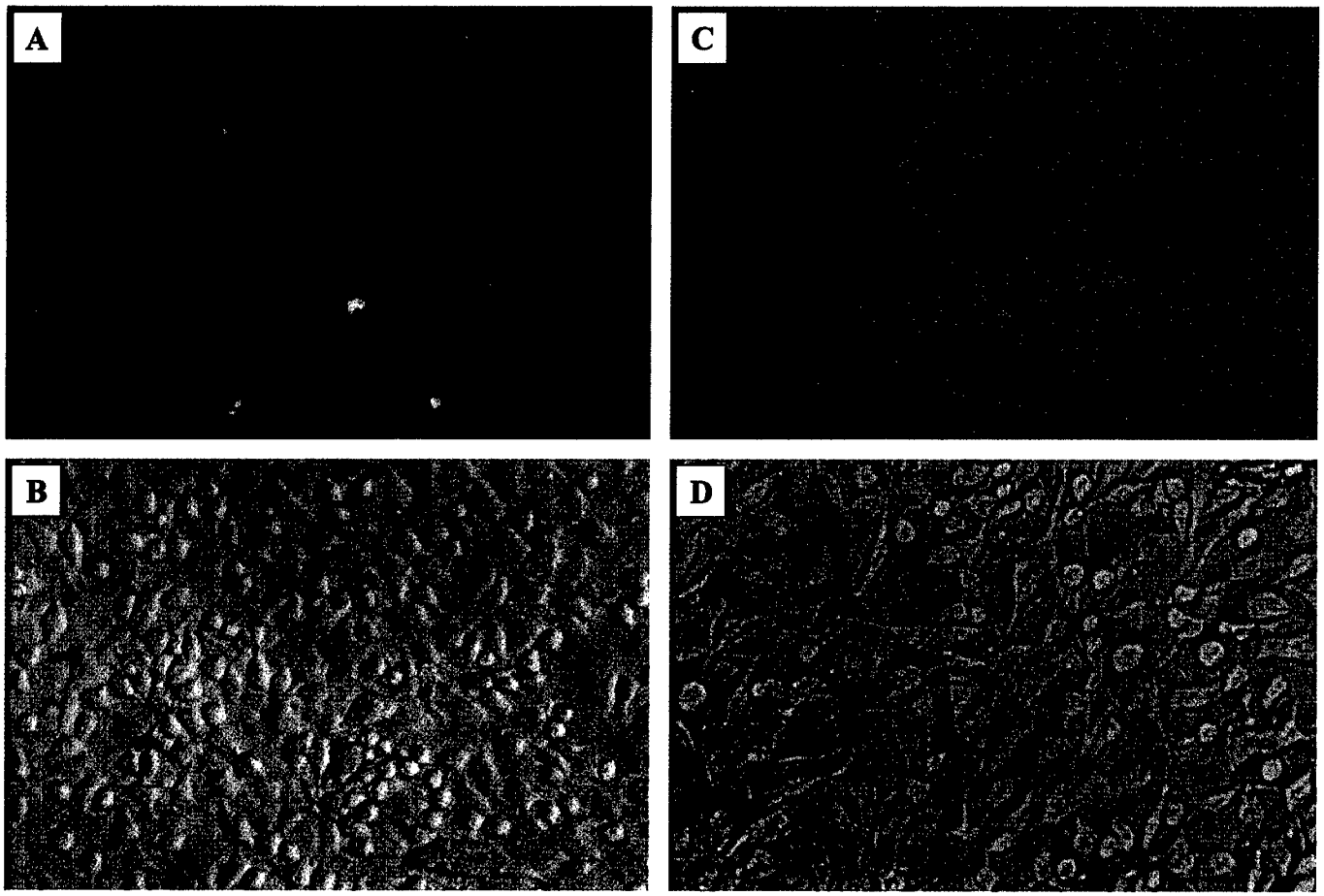

Fig. 1. Visualization of αGal expression in MC38 colon carcinoma cells and lack of expression in αGal-negative B16.BL6-2 melanoma cells using FITC-IB4 lectin staining. Cells were incubated with a 1:50 dilution of FITC-IB4 lectin in Opti-MEM at room temperature for 15 min. *A* and *B* show MC38 cells, and *C* and *D* show B16.BL6-2 cells. FITC-lectin staining of the αGal moiety in the outer cell surface membrane demonstrates the activity of the αGT gene in MC38 cells and lack of gene expression in B16.BL6-2 cells. *A* and *C* were photographed using a Nikon DM505 FITC cube, and *B* and *D* were photographed under brightfield light using the same FITC cube.

A^{450} absorbance of >0.1 above background were considered positive. The average titer of anti-αGal antibody in mice immunized one time with RRBC was 1:1600 (Fig. 3*A*). Mice that were immunized twice with RRBC had an average titer of 1:8000 (Fig. 3*B*). The immune response in αGT KO mice to αGal was determined by collecting serum from mice at various times after RRBC immunization on days 14 and 28. An anamnestic IgG immune response to αGal that peaks at 7 days after the second immunization was observed (Fig. 4*A*).

Serum samples collected on day 0 from αGT KO mice immunized once on day −14 (14 mice) or twice on days −28 and −14 (8 mice) were assayed for their antibody isotype. An A^{450} absorbency > 0.2 for an individual antibody isotype was considered positive. Serum was negative for an antibody isotype and subclass if the A^{450} absorbance was <0.2. RRBC immunization stimulated the production of IgM, IgG1, and IgG3 antibodies (Fig. 4*B*). The predominant subclass was IgG3, which developed in 50% (11 of 22 immunized mice). The IgG1 subclass was observed with higher frequency (36%) then either IgG2a or IgG2b (5 and 13%). All mice immunized regardless of schedule demonstrated high levels of IgM antibody, and no immunized mice demonstrated any IgA antibody isotype. Interestingly, all immunized mice developed antibody with κ light chain, and no λ light chain was found in these mice.

Tumor Cell Challenge. Fourteen days after RRBC immunization of αGT KO mice and mock immunization of αGT KO and C57BL/6 controls, the mice were divided into three groups and implanted i.p. with different dilutions of MC38 colon carcinoma cells in a blinded experiment. Tumor cell dilutions were made, coded, and randomized before injection into mice. All mice were observed daily for tumor development and were euthanized when tumors reached ~1200 mm^3, exhibited ascites fluid production, or when the mice were moribund.

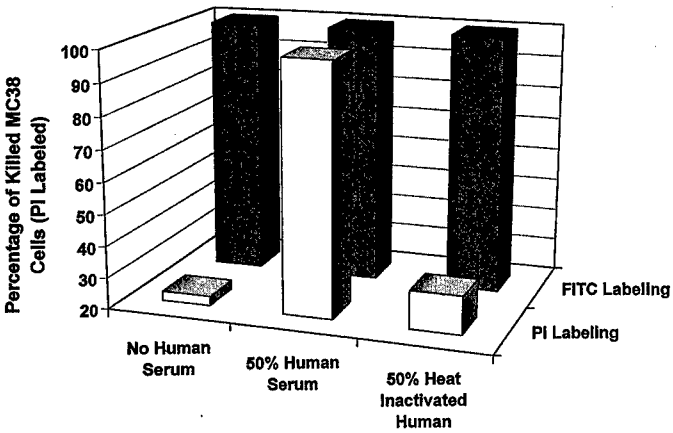

Fig. 2. αGal-positive MC38 colon carcinoma cells are killed when exposed to serum containing active human complement. MC38 cells were stained with FITC-IB4 lectin and suspended in test media containing no human serum, 50% human serum, and 50% heat-inactivated human serum. After 1-h incubation at 37°C, cells were incubated with a suspension of PI for 5 min at room temperature. Flow cytometry demonstrated that only serum containing active complement was able to kill αGal expressing MC38 cells.

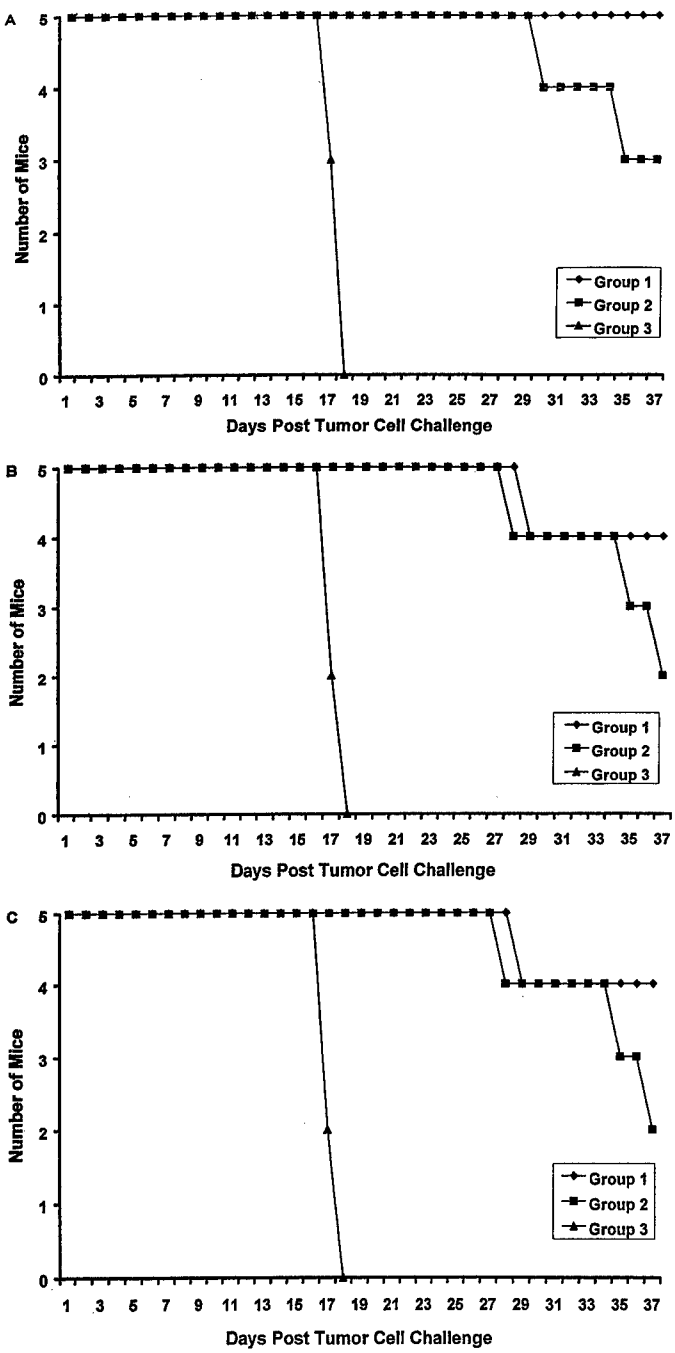

Fig. 5. Mice immunized with RRBC showed superior protection to tumor challenge with αGal-positive MC38 colon carcinoma cells compared with mock-immunized mice. Survival curves for immunized αGT KO mice and mock-immunized αGT KO mice and C57BL/6 mice after MC38 tumor cell challenge demonstrate that an immune response to αGal epitopes can increase the survival of immunized mice in comparison to mice that are not immunized with αGal. Group 1 consisted of αGT KO mice that were immunized one time with 10^7 RRBC before challenge 14 days later with MC38 cells. Group 2 consisted of αGT KO mice that were mock immunized and challenged 14 days later. Group 3 consisted of C57BL/6 mice that were mock immunized and challenged 14 days later. Three dilutions of MC38 cells were used to challenge mice (*A*) 2.5×10^4 MC38 cells, (*B*) 5.0×10^4 MC38 cells, or (*C*) 1.0×10^5 MC38 cells. Mock-immunized C57BL/6 mice all died or were euthanized within 18 days. Mock-immunized αGT KO mice showed 40–60% survival. Immunized αGT KO mice showed 80–100% survival ($P = 0.031$).

cancer cell line was chosen for this study. Colon cancer is the third most common form of cancer and third leading cause of death (4).

The αGT KO mouse is an ideal small animal model to study our hypothesis. The loss of the αGT gene by these mice mimics the evolutionary loss of this gene by ancestral Old World primates and humans (5). αGT KO mice produce little or no αGal-specific antibody (25, 26) but are able to develop an immune response to αGal when immunized with RRBCs. These mice can produce αGal-specific antibody with high titers and specificity in some animals similar to those observed in humans (27). The RRBC immunization protocol we used with the αGT KO mice resulted in high titers of anti-αGal antisera that could be detected by indirect ELISA at a 1:16,000 dilution. Previously, LaTemple *et al.* (34) demonstrated a partially protective immune response when αGT KO mice are vaccinated with αGal-expressing B16 cells (after stable transfection of αGal-negative B16 cells with αGT cDNA) and challenged with parental B16 cells.

MC38 murine colon carcinoma cells have a functional αGT enzyme and express αGal on their cell surface glycoproteins. Fig. 1 shows the difference in αGal expression between MC38 colon carcinoma and B16.BL6-2 melanoma cells used in this study. The αGal moiety expressed on the surface of MC38 cells is labeled brightly with

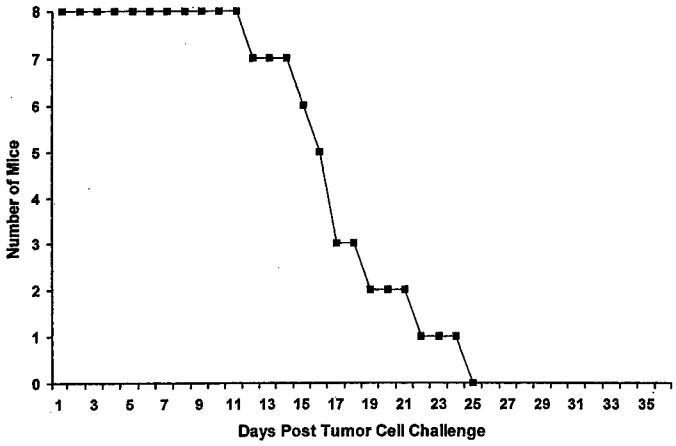

Fig. 6. Mice immunized with RRBC were not protected after tumor challenge with αGal-negative B16.BL6-2 melanoma cells. Survival curve for immunized αGT KO mice after challenge with B16.BL6-2 cells shows that an immune response to αGal epitopes does not prevent tumor growth and death. Mice were immunized one time with 10^7 RRBC and challenged 14 days later with 1.0×10^5 B16.BL6-2 melanoma cells. None of the mice survived tumor challenge despite immunization with αGal antigen because the B16.BL6-2 tumor cell line is a non-αGal-expressing cell.

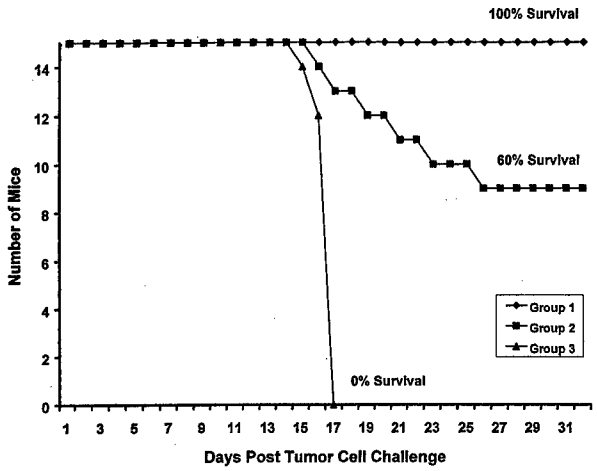

Fig. 7. Mice immunized twice with RRBC showed superior protection to tumor challenge with αGal-positive MC38 colon carcinoma cells compared with mock-immunized mice. Survival curves for immunized αGT KO mice (group 1) and mock-immunized αGT KO mice (group 2) and C57BL/6 mice (group 3) after MC38 tumor cell challenge. Mice were immunized or mock immunized twice with 10^7 RRBC, 28 and 14 days before tumor cell challenge. All mice were challenged with 2.5×10^4 MC38 cells. Group 1 αGT KO mice were immunized with RRBC and demonstrated 100% survival to tumor cell challenge ($P < 0.0069$) in comparison to group 2 mock-immunized αGT KO mice (60% survival) and group 3 mock-immunized C57BL/6 mice (0% survival).

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High-Throughput Fluorescent Screening of Transgenic Animals

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Key Words: high-throughput, screening, phenotyping, genotyping, transgenic, knockout, ⟨(1,3)galactosyltransferase

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diluted with 85 ∝l Hanks Balances Salt Solution (HBSS, Invitrogen-Life Technologies Inc., Carlsbad, CA). Samples were stained by adding 100 ∝l of 1 ∝g/ml biotinylated anti-⟨gal *Griffonia simplicifolia* IB4 isolectin (Vector Laboratories, Burlingame, CA) diluted in HBSS and incubated at room temperature for 30 min. The biotinylated IB4 isolectin was detected with 0.5 ∝g/ml avidin conjugated PE (BD Pharmingen, San Diego CA). Cells were pelleted by centrifugation for 5 min at 423 x g using a 96-well plate holder followed by washing with 200 ∝l HBSS, and pelleted as before. Cells were resuspended in 200 ∝l HBSS and 50 ∝l were transferred to a flat-bottom 96-well plate (Corning Co). The plate was centrifuged as above and microscopically analyzed (Nikon Diaphot) using a Cy3 (532 nm) fluorescent filter (Table1).

Validation of the ⟨gal Phenotype

Genomic DNA was isolated from wild-type and ⟨GT KO blood samples. Polymerase chain reaction (PCR) screening of ⟨GT KO was performed using the the forward primer (GALFDJH) 5'-GAG CAC ATC CTG GCC CAC ATC CAG CAC GAG-3', which binds upstream of the *SalI* site in exon 9 of the ⟨gal gene, and the reverse primer (NeoU) 5'-GGT GGA GAG GCT ATT CGG CTA TGA – 3' which binds the 5' region of the neo gene. To screen the wild-type, PCR was performed with the GALFDJH primer and the reverse primer (BGLGAL#2) 5'-AGA TCT TCA GAC ATT ATT TCT AAC CAA ATT ATA CTC-3' which binds 481 bp downstream of the *SalI* site. To construct the ⟨gal knockout strain, Thall et al., inserted the pGKneo construct into the *SalI* site of exon 9 of the ⟨gal gene (10). Therefore, 751 bp and 481 bp PCR products from ⟨GT KO and wild-type mice, respectively, was expected. A PCR program of 94oC for 30 sec, 55oC for 90 sec, and 68oC for 60 sec for a total of 40 cycles was used.

DISCUSSION

With the use of transgenic animals many fields in biology have made rapid advances that were not as easily achievable before the development of this technology. However, the production and screening of these animals is far from streamlined and is therefore laborious and slow. Techniques designed to enhance the process and save the investigator time and resources are in demand. Therefore, we have developed a novel high-throughput phenotyping technique that allows the investigator to determine putative transgenic homozygotes from WBC's using direct or indirect fluorescent microscopy. Either a primary labeled fluorescent probe such as an antibody or lectin, or an unlabeled probe followed by a secondary detection system can be used to screen large numbers of samples in a microtiter plate within a short period of time. By contrast, other phenotyping methods, such as flow cytometry, may be suboptimal for many reasons. First this method requires a larger volume of blood to be collected from the animal which can be particularly difficult in mice. Subsequent sample collection may be needed to complete the validation steps. Second, sample analysis is slower due to the necessity of handling individual tubes for each blood sample and acquiring the flow data for each tube. Finally, a highly training staff member must be capable of calibrating the machine and acquiring the data for each sample. Using flow cytometry, therefore, may be useful for some specialized applications, but for large scale screening, may be inadequate. In our lab, we found the limiting step using flow cytometry was the number of samples that could be analyzed in a day. Using our high-throughput screening technique, we found that the limiting factor was how many animals could be bled in a day, rather than analyzed. For example, using flow cytometry, at least 2 days of sample preparation and analysis were required to phenotype approximately 30 animals. By comparison our rapid screening method required only 1 day for sample preparation and analysis of over 80 animals. Moreover, unlike the flow cytometry method, we could use the remaining blood samples of putative homozygotes to extract genomic DNA from the WBC's to validate the phenotype without having to collect more tissue from the animals.

We have used this method in our lab to screen for the ⟨gal null phenotype in 363 F1 offspring from F0 heterozygote CD1 KO x ⟨GT KO crossed mice. In these experiments we collected blood from each of the animals and used a variation of the protocol in table 1 that allowed us to store blood samples long term. Samples were fixed and simultaneously the red blood cells were lysed prior to FITC conjugated IB_4 isolectin labeling. Seventy-nine animals were found to be ⟨gal negative and 27 were tested for the double knockout phenotype of CD1$^{-/-}$ and ⟨gal$^{-/-}$ resulting in 3 males and 7 females. Results of genotyping these mice by PCR using genomic DNA extracted from the blood samples validated the phenotype observed using the fluorescent screening technique.

Many protocols require the use of a variety of genetic backgrounds from which to produce transgenic animals. For example, the ⟨GT KO mice were a cross between C57/BL6, DBA/2, and 129SV backgrounds. For immunological studies, haplotype matched mice are desirable. Therefore, in addition to phenotyping

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Manuscript #1 -- **Characterization of a gastrointestinal stromal tumor line derived from an α(1,3)galactosyltransferase knockout mouse and its evaluation as a potential gene therapy model**

Figures 1 through 6 supplied in order with legends on separate pages.

TABLE AND FIGURE LEGENDS

Table 1. Various combinations of chemicals used to induce tumors in αGT KO mice. Mice were injected i.p. twice fourteen days apart with 250 μl of drug dissolved in olive oil.

Figure 1. a. Frozen section of primary tumor, CA320M, derived from the gastrointestinal tract of a female mouse, 16x. Inset shows morphologies consistent with a sarcoma, 40x. b. Paraffin section of tumor arising from transplanted cultured CA320M cells injected s.c. in the abdomen of a female H-$2^{b/b}$ haplotype mouse, 16x. Inset shows mitotic figures at 40x magnification.

Figure 2. Flow cytometric analysis of CA320M haplotype. Antibodies against H-2Kb, H-2Kd, H-2Db, and H-2Dd were used on 10^6 cells. The histogram in the left panel shows dual labeled cells for the H-2d haplotype. The histogram in the right panel shows dual labeled cells for the H-2b haplotype. These data indicate that CA320M was derived from a homozygous H-$2^{b/b}$ mouse.

Figure 3. Cell cycle analysis of nonsynchronized CA320M and B16.BL6 cultured in normal serum (D10) or serum starved conditions (D0.1). a. The change in S phase of CA320M in both culture conditions from 0 to 48 hr. b. The changes in G1 of CA320M for the same conditions. c. The changes in S phase of B16.BL6 d.

The changes in G1 phase of B16.BL6 for the same conditions. [◆] Cells cultured in D10, [■] Cells cultured in D0.1. Both CA320M and B16.BL6 appear to arrest in G1 by 48 hr in D0.1 as indicated by an increase in percent G1 with a concomitant decrease in S phase. e. Modeling the proliferation rate of CA320M. [O] Theoretical curve of 2 cell divisions in 24 hr, [◆] Cells cultured in D10, [■] Cells cultured in D0.1, [Δ] Theoretical curve of 3 cell divisions in 24 hr. Cells double approximately every 12 hr in D10. f. Percent CFSE positive cells and the number of times they doubled in 48 hr in D10 and D0.1 cultures. Cells cultured in D10 cycled 3-4 times versus 2-3 times in D0.1.

Figure 4. a. CA320M unmodified cells stained with FITC labeled IB_4 lectin specific for αgal epitopes. b. Cells transduced with HE7αgal1 for 8 hr showing the presence of cell surface αgal epitopes. c. Cells transduced with HE7EGFP as a control of efficiency of viral infection showing GFP fluorescence. d. Bright field of a representative culture of CA320M. 40x magnification.

Figure 5. Serum killing analysis with a. normal human serum (NHS) (histograms on the right are of HE7αgal1 expressing cells gated on 7AAD and C3c staining) or b. serum collected from mice (MS) primed against the αgal epitope by 2 injections of RWB (histograms on the right are similar to above). Two hundred thousand cells were incubated with 50% serum for 1hr at 37°C and tri-labeled for

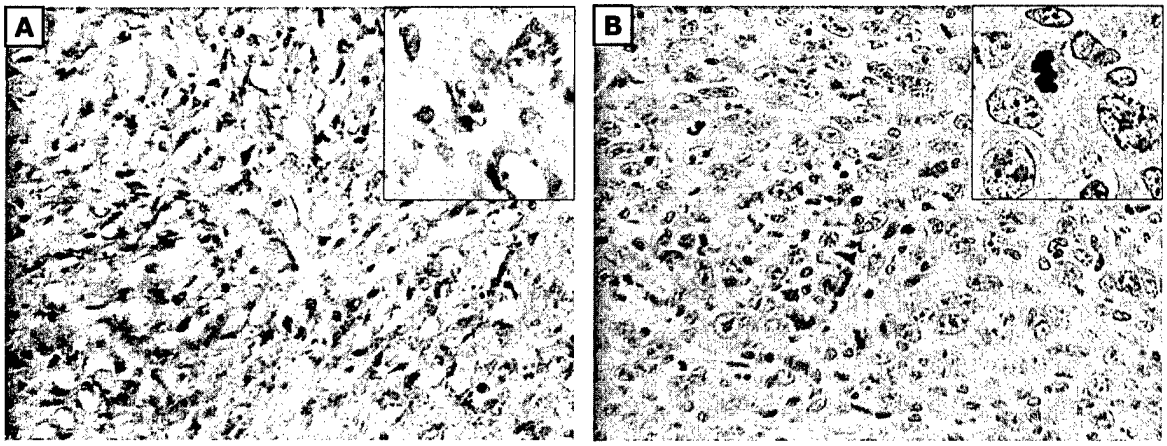
αgal epitope, C3c complement, and cell death discrimination by 7AAD. Results are plotted as percent αgal positive C3c bound dead cells except CA320M which are only C3c/7AAD positive. HIS, Heat inactivated serum.

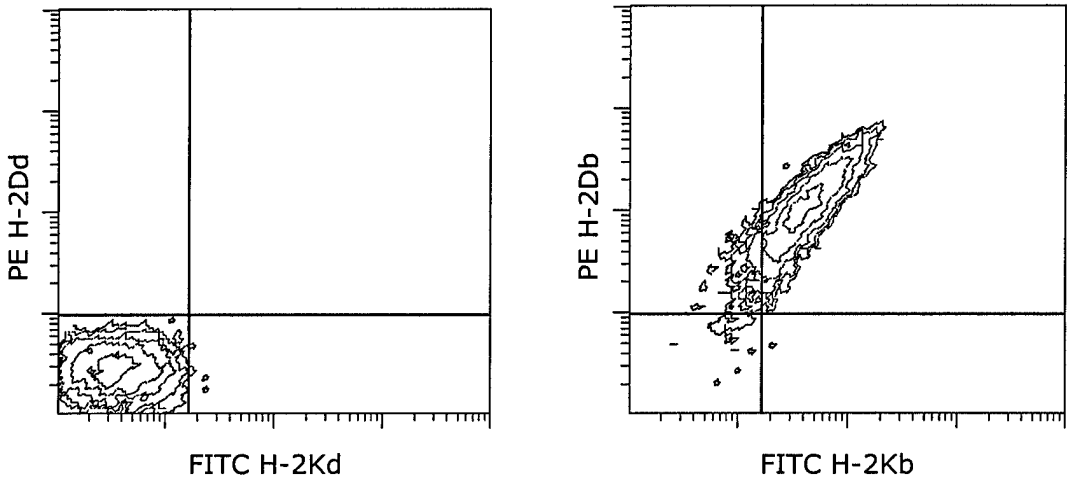
Figure 6. *In vivo* tumor growth kinetics of CA320M in αGT KO female H-$2^{b/b}$ mice. Mice (n = 8 per group) were injected subcutaneously with cells suspended in 100 µl HBSS. a. Mean (± SEM) tumor volume over time, [■] $1x10^6$ or [◆] $5x10^6$ cells per animal. Percentage of mice permitting tumor growth through day 30. c. Percentage of mice with lethal tumor volume of over 400 mm^3 by day 30.

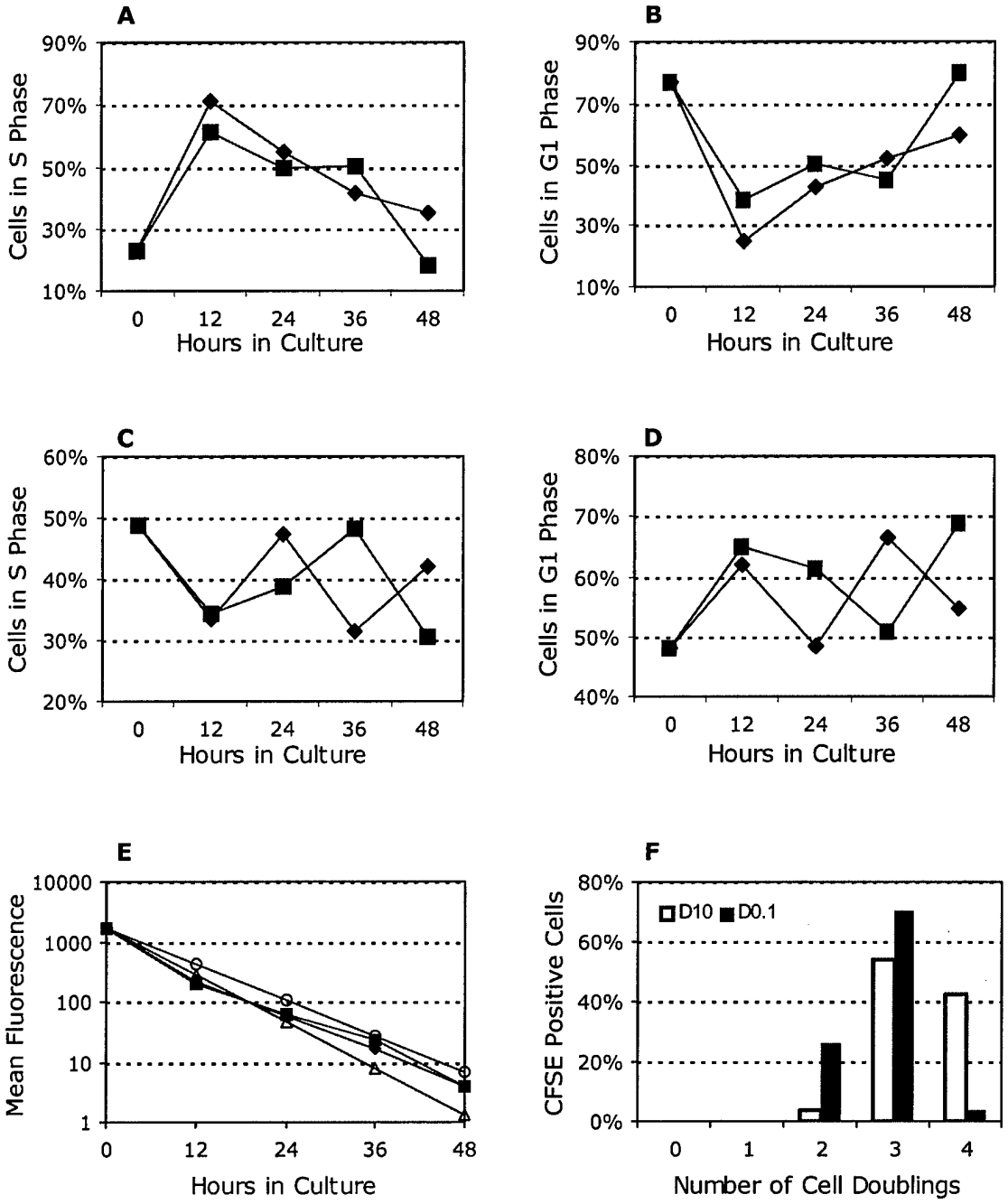
Table 1. Drug Combinations and Resulting Tumors

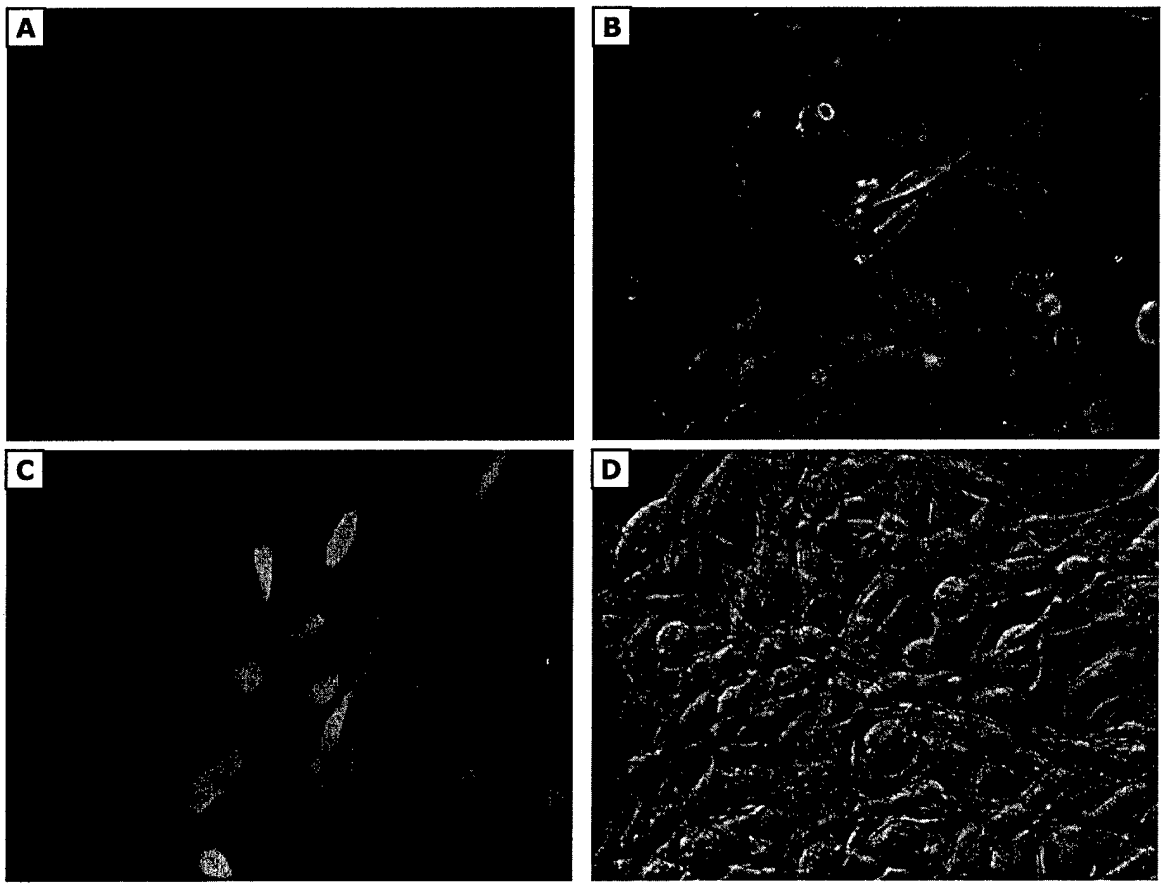
DMBA (mg)	3-MC (mg)	MHP (mg)	No. of Tumors*
0.5	1.0	n/a	8
1.0	1.0	n/a	8
2.0	1.0	n/a	9
2.0	0.1	n/a	6
2.0	0.5	n/a	8
n/a	n/a	1.0	5

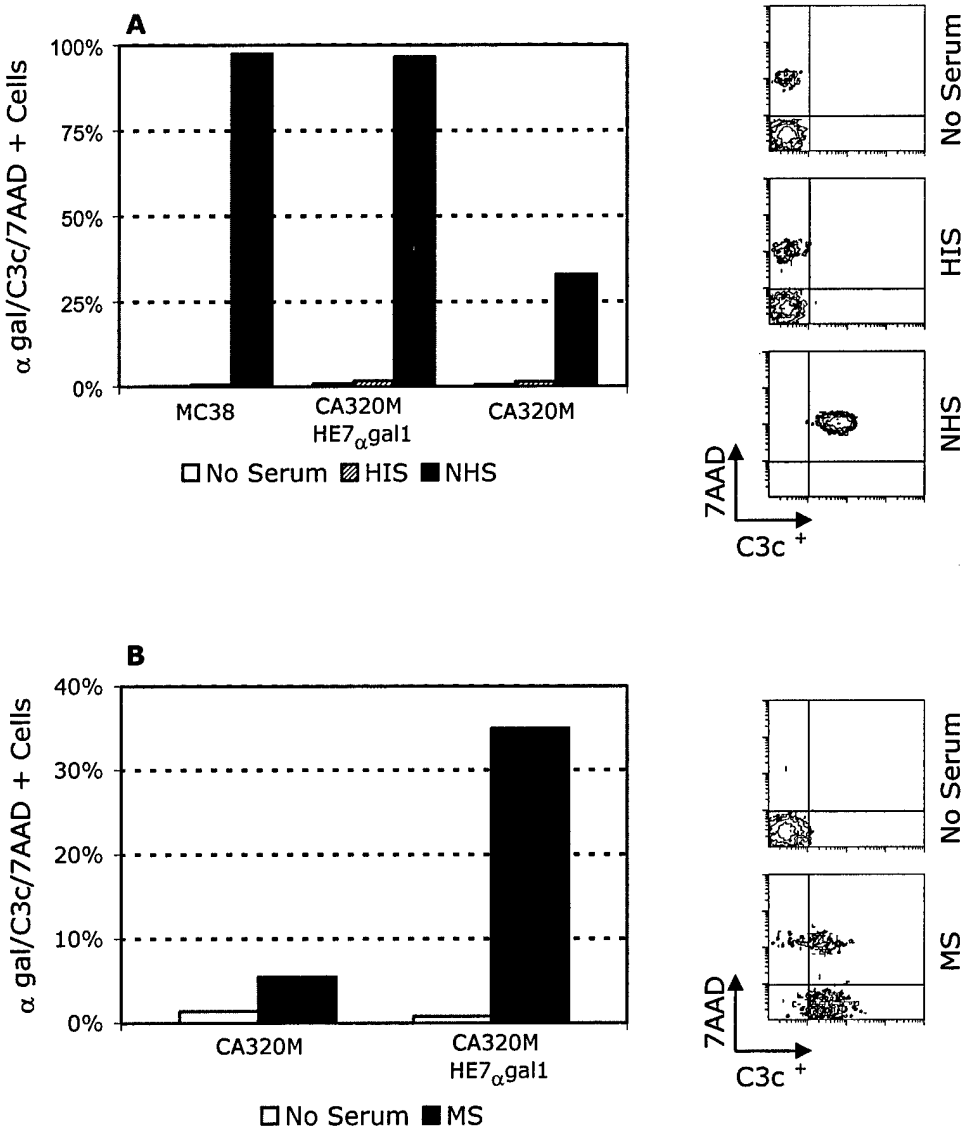
* Number of animals with at least one tumor
DMBA; 9,10-dimethyl-1,2-benz-anthracene
3-MC; 3-methylcholanthrene
MHP; 6α-methyl-17α-hydroxy-progesterone acetate
Chemicals dissolved in 250 μl olive oil inj'd twice i.p.

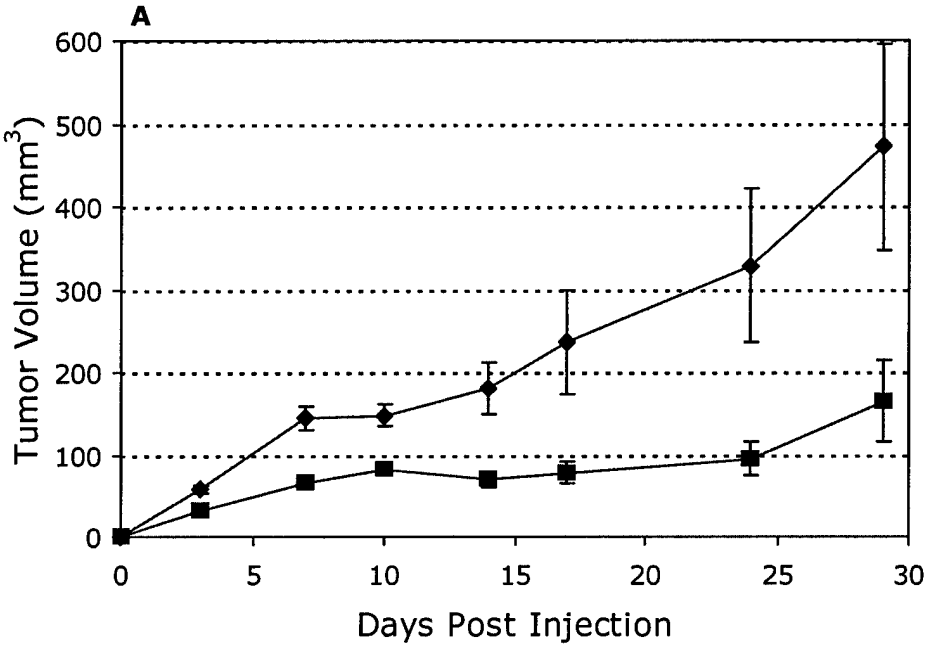

A
B

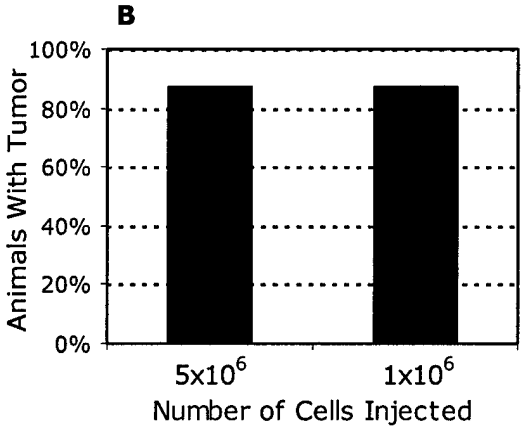

PE H-2Dd
FITC H-2Kd
PE H-2Db
FITC H-2Kb

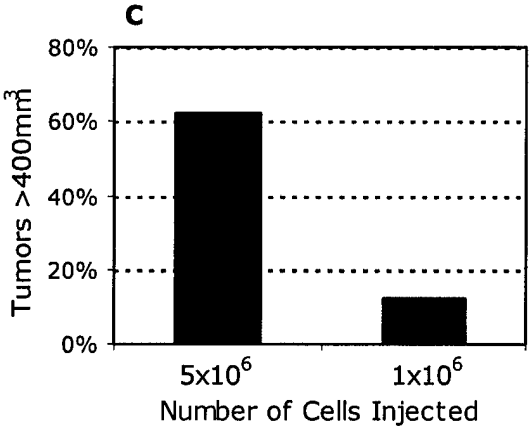

A
90%
70%
50%
30%
10%
Cells in S Phase
0
12
24
36
48
Hours in Culture
B
90%
70%
50%
30%
10%
Cells in G1 Phase
0
12
24
36
48
Hours in Culture
C
60%
50%
40%
30%
20%
Cells in S Phase
0
12
24
36
48
Hours in Culture
D
80%
70%
60%
50%
40%
Cells in G1 Phase
0
12
24
36
48
Hours in Culture
E
10000
1000
100
10
1
Mean Fluorescence
0
12
24
36
48
Hours in Culture
F
80%
60%
40%
20%
0%
D10
D0.1
CFSE Positive Cells
0
1
2
3
4
Number of Cell Doublings


A
B
C
D


A
α gal/C3c/7AAD + Cells
100%
75%
50%
25%
0%
MC38
CA320M HE7αgal1
CA320M
No Serum
HIS
NHS
7AAD
C3c +
B
α gal/C3c/7AAD + Cells
40%
30%
20%
10%
0%
CA320M
CA320M HE7αgal1
No Serum
MS
7AAD
C3c +


A
600
500
400
300
200
100
0
Tumor Volume (mm3)
0
5
10
15
20
25
30
Days Post Injection


B
100%
80%
60%
40%
20%
0%
Animals With Tumor
5x10^6
1x10^6
Number of Cells Injected


C
80%
60%
40%
20%
0%
Tumors >400mm3
5x10^6
1x10^6
Number of Cells Injected

Manuscript #2 – **High Throughput Fluorescent Screening of Transgenic Animals**

Table and Figures supplied in order with legends on separate preceding page.

TABLE AND FIGURE LEGENDS

Table 1. High-Throughput Phenotyping Protocol. Peripheral blood cells are labeled with a fluorescent probe specific for the antigen corresponding to the transgenic genotype and viewed under fluorescent microscopy.

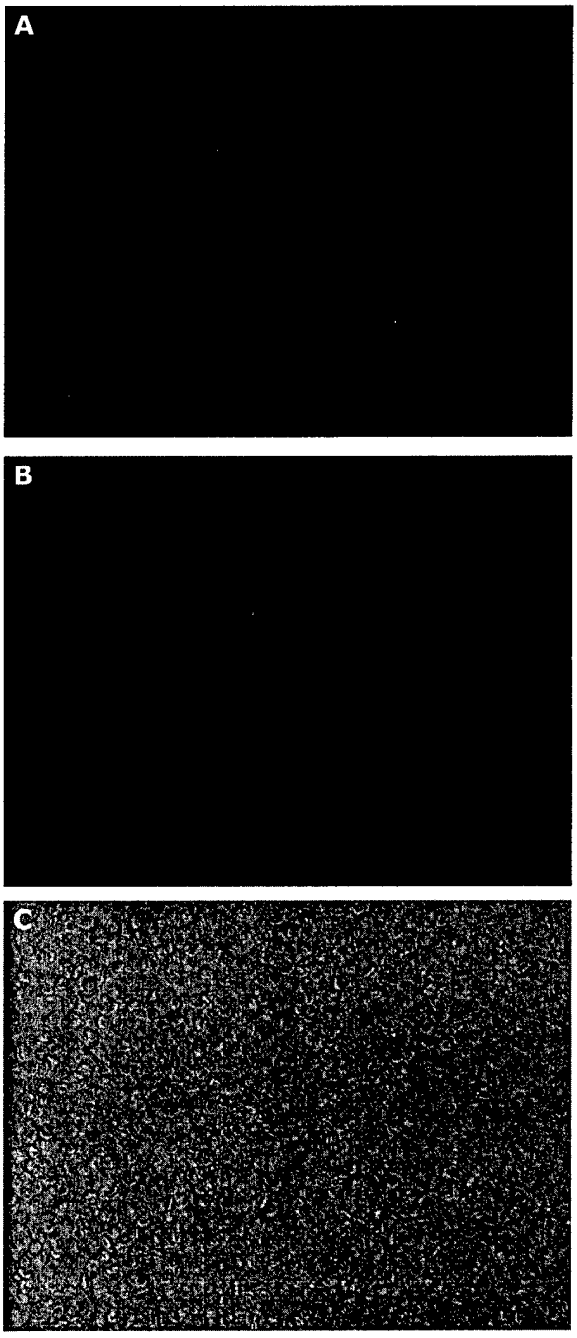
Figure 1. a. PE labeled anti-αgal IB4 isolectin successfully detected αgal epitopes on CD1 KO mouse blood cells. b. ⟨GT KO mice do not express the αgal gene as confirmed by the lack of anti-αgal IB4 isolectin staining of blood cells. c. Bright field photo representing the number of cells used in this protocol.

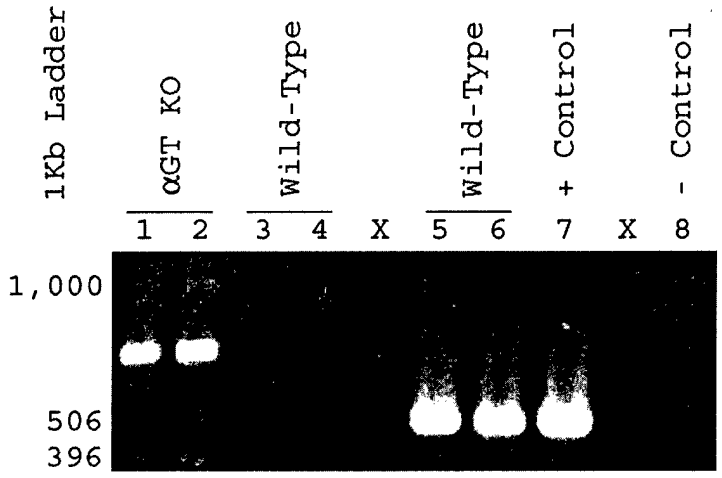
Figure 2. PCR results after amplifying a region of exon 9 from the αgal gene using genomic DNA extracted from blood samples. 1 Kb DNA ladder was used as the marker. Lanes 1 and 2 are from αGT KO mice showing the expected 751 bp fragment corresponding to the αgal specific amplification of the neo used to disrupt the αgal gene. Lanes 3 and 4 are from wild-type mice demonstrating the absence of neo in the αgal gene. Lane 5 and 6 demonstrate wild-type exon 9 of the αgal gene showing the expected 481 bp band. Lane 7 is the positive control from a cDNA clone of the αgal gene showing the expected 481 bp fragment. Lane 8 is the negative control where all three primers were used in the reaction without template DNA.

Table 1

1. Collect ~ 200 µl whole blood in heparinized tubes.
2. Dilute 15 µl blood with 85 µl HBSS in 96-well round bottom plate.
3. Mix samples with 100 µl of fluorescently labeled probe or unconjugated probe*. Incubate 30 min RT.
4. Pellet cells by centrifugation 5 min @ 423 x g, wash with 200 µl HBSS, Repeat 3 with secondary fluorescently labeled probe (or go to step 5), pellet cells again and wash.
5. Raise in 200 µl HBSS, transfer 50 ∝l to 96-well flat bottom plate, cytospin as in step 4 and view under fluorescent microscope.

Table 1. High-Throughput Phenotyping Protocol
* For primary labeled probes such as antibodies or lectins, do not repeat step 3. For some applications cells may need to be fixed and/or permeabilized for the probe to bind. To increase the number of blood cells or to detect more rare targets lyse RBC's prior to staining.


A
B
C


1Kb Ladder
αGT KO
Wild-Type
Wild-Type
+ Control
- Control
1 2 3 4 X 5 6 7 X 8
1,000
506
396